

Mechanisms Involved in the Cell Death in PC-12 Cells and Chicken Cerebellar Neurons under Serum-deprivation, Bisphenol-A Exposure and MEHP Toxicity

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Abstract

Environmental estrogens are long known to interfere with neurological, reproductive, cardiovascular and musculoskeletal system of human-beings and animals. Chemicals like bisphenol-A and phthalates are just two of many environmental endocrine disruptors that have deleterious effects on the health. Earlier studies on neuronal cultures in rats have shown that both bisphenol-A and phthalates induce reactive oxygen species and apoptosis. Mitogen-activated protein kinase pathway is reported to be involved in the apoptotic signaling. One part of this study is to explore the effects of these chemicals and the mechanisms involved in their toxicity.

The research has been done on two alternative cell culture models: PC-12 cells and chicken cerebellar granule neurons. Chicken neurons have given opportunity to investigate toxicity at different stages of neuronal development in vitro. Vitamins C and E, which are ROS scavengers showed protection after bisphenol-A exposure at the early stages of neuron development. Chicken neurons were more sensitive to bisphenol-A and phthalate exposure than PC-12 cells.

Since serum-deprivation has been widely used to initiate apoptosis in the PC-12 cells, this model was used as comparison. ROS is known to be generated as a result of such exposure which in turn consumes endogenous glutathione under oxidative stress. Since glutathione is a vital endogenous antioxidant, measuring glutathione has given a better understanding to mechanisms behind the cell death and ROS production. The current study showed that glutathione levels fell after deprivation or when α -estradiol, but not vitamins C and E, was added to the serum-deprived PC-12 cells in accordance with earlier promoter study. However, bisphenol-A and MEHP did not change glutathione level.

In conclusion, whereas deprivation induces cell death with reduction in GSH level, bisphenol-A and phthalate induce cell death with no effect on glutathione level.

Contents

<i>Acknowledgements</i>	2
<i>Abstract</i>	4
<i>Contents</i>	7
<i>Abbreviations</i>	12
<i>1. Introduction</i>	15
1.1 Environmental toxins.....	15
1.1.1 Bisphenol-A.....	16
1.1.2 Phthalates.....	17
1.2 ROS.....	17
1.3 Oxidative stress.....	18
1.4 Deprivation and apoptosis.....	18
1.5 MAP-kinase.....	19
1.6 Caspase-3.....	21
1.7 Glutathione an endogenous antioxidant.....	22
1.8 Antioxidants.....	25
1.9 PC-12 cells cell line.....	26
1.10 Chicken cerebellar granule neurons.....	26

1.11	Aim of the study.....	28
2	<i>Materials and Methods</i>.....	29
2.1	Splitting of PC-12 cells.....	29
2.2	Deprivation of PC-12 cells.....	30
2.3	Treating PC-12 cells with antioxidants.....	31
2.4	Cell death count for PC-12 cells.....	32
2.5	Making of trypan blue.....	32
2.6	Bisphenol-A concentrations.....	33
2.7	Phthalate concentrations.....	34
2.8	Harvesting cells for glutathione measurement.....	35
2.9	Glutathione kit one (ApoGSH detection kit).....	35
2.9.1	Cell lysis buffer versus RIPA buffer in glutathione test measurement.....	36
2.10	Glutathione kit two.....	35
2.10.1	Making of PBC.....	38
2.10.2	Glutathione standard dilutions by serial dilutions.....	41
2.11	Protein measurement.....	44
2.11.1	Solutions for protein measurement.....	45
2.11.2	Application of the standard.....	45
3	<i>Results</i>.....	48

3.1	Glutathione levels in PC-12 cells.....	48
3.1.1	Glutathione level in control and deprived PC-12 cells.....	49
3.1.2	Glutathione levels in control with antioxidants.....	50
3.1.3	Glutathione levels in deprived PC-12 cells with antioxidants.....	52
3.1.4	Glutathione levels after addition of exogenous glutathione in PC-12 cell.....	54
3.1.5	Glutathione levels in PC-12 cells after administration of exogenous glutathione in serum-free medium.....	55
3.1.6	Mechanisms behind the regulation of glutathione levels in control PC-12 cells.....	57
3.1.7	Mechanisms behind regulation of glutathione levels in deprived PC-12 cells.....	60
3.1.8	Mechanism behind the fall of glutathione levels in PC-12 cells when α -estraiol is added.....	62
3.2	Bisphenol-A in PC-12 cells.....	65
3.2.1	Dose-response of bisphenol-A in PC-12 cells.....	65
3.2.2	Mechanisms behind bisphenol-A toxicity in PC-12 cells.....	67
3.2.3	Glutathione levels under exposure to bisphenol-A in PC-12 cells.....	69
3.3	Phthalate in PC-12 cells.....	71
3.3.1	Dose-response in PC-12 cells after phthalate exposure.....	71

3.3.2	Mechanisms behind phthalate toxicity in PC-12 cells.....	74
3.3.3	Glutathione levels in PC-12 cells after phthalate exposure.....	76
3.4	Chicken neurons.....	78
3.4.1	Dose-response in chicken neurons under bisphenol-A exposure.....	78
3.4.2	Mechanisms of bisphenol-A toxicity in chicken neurons.....	80
4	<i>Discussion</i>.....	82
4.1	Discussion of cell models of study.....	82
4.1.1	Use of PC-12 cell line to study neurons.....	82
4.1.2	Use of cerebellar granule chicken neurons to study neurons.....	83
4.2	Effect of antioxidants on glutathione levels in PC-12cells.....	85
4.2.1	Effect of vitamins on glutathione level in PC-12 cells.....	85
4.2.2	Effect of substitution of endogenous glutathione by additional exogenous glutathione on glutathione level in PC-12 cells.....	86
4.2.3	Effect of α -estradiol in serum-deprived PC-12 cells.....	88
4.3	Bisphenol-A toxicity in PC-12 cells and cerebellar granule neurons.....	89
4.3.1	Effect of bisphenol-A.....	89
4.3.2	Uncovering mechanisms behind bisphenol-A toxicity.....	89
4.3.3	Effect of bisphenol-A on glutathione level.....	91
4.4	Effect of phthalate toxicity on PC-12 cells.....	92

4.4.1	Effect of MEHP in PC-12 cells.....	92
4.4.2	Uncovering mechanism behind phthalate toxicity in PC-12 cells.....	92
4.4.3	Effect of phthalate on glutathione level in PC-12 cells.....	93
4.5	Constrains and evaluation of bisphenol-A and phthalate toxicity.....	94
4.6	Conclusion.....	96
	<i>References.....</i>	98
	<i>Chemicals and biological products.....</i>	107

Abbreviations

ANOVA	analysis of variance
AIF	apoptosis-inducing factor
AP-1	activator protein-1
ARE	antioxidant response element
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma protein 2
BME	basal medium eagle
BPA	bisphenol-A
BSA	bovine medium albumin
CLB	cell lysis buffer
CNS	central nervous system
DMEM	dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GSH	reduced glutathione

GSSG	oxidized glutathione
HS	horse serum
γ -GCS	γ -glutamylcysteine synthetase
JNK	jun-N terminal kinase
MAP	mitogen activated protein
MAPK	mitogen-activated protein kinase
MEHP	mono(2-ethylhexyl) phthalate
MEK	MAPK/ERK kinase
NF-kB	nuclear factor-kB
NGF	nerve growth factor
PBS	phosphate buffered saline
ROS	reactive oxygen species
SD	standard deviation
SSA	5%5-sulfosalicylic acid
α T	α -tocopherol
Vitamin C	ascorbic acid
Vitamin E	α -tocopherol
WR	working reagent

1 Introduction

1.1 Environmental toxins

When asserting any chemical compound, be it a toxin or antioxidant, it is assured that both are toxic. "All things are poison and nothing is without poison, only the dose permits something not to be poisonous."

Paracelius

The quote emphasizes that any chemical is poisonous and only the dose is the one to define the toxicity of the compound.

Environmental pollutants have been reported to disturb the antioxidant balance of the cells by causing oxidative stress (Bindhumol et al., 2003).

Both Bisphenol-A and phthalates are hormone- endocrine disruptors and are known to induce ROS (see 1.2). These endocrine-disrupting compounds are contaminants of the environmental system, and these chemicals do alter and effect the reproductive system not only of animals, but humans too (Watson et al., 2010). The current research in science tries to elucidate the potential ecological problem and physiological impact on the organisms. Xenoestrogens have been reported to interfere in the hormonal way during different life-stages both for women and men in reproductive, cardiovascular, respiratory, neurological way (Watson et al., 2010). Therefore it is important to concentrate on these xenoestrogens that have a gradual bioaccumulation.

1.1.1 Bisphenol-A

Bisphenol-A is a monomer, of epoxy resins and polycarbonate plastics (Bindhumol et al., 2003). Bisphenol-A is a common material that is found in cans, dental fillings, toys, food packaging and is widely spread in the environment. Bisphenol-A was reported earlier to produce reactive oxygen species (ROS) in the liver of male rats (Bindhumol et al., 2003). This study has also inferred that oxidative stress appeared as a main disturbance, while the activities of the antioxidant enzymes decreased rapidly. This finding alerts the people as low toxicities of bisphenol-A can cause harmful effects by accumulation in the environment or in the organism. Superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase have decreased its activity (Bindhumol et al., 2003). Earlier experiment on the PC-12 cells and cortical neuronal cells has revealed that bisphenol-A exposure above 50 μ M does in fact promote apoptosis, and decreases cell viability (Lee et al., 2007). Bisphenol-A has adverse effects through respiration or through skin exposure. Reproductive system, kidney, liver, DNA, CNS have been effected under bisphenol-A exposure.

It is believed that c-Jun N- terminal kinases and signaling regulating kinases were activated by phosphorylation under bisphenol-A exposure in hippocampal neuronal cells (Lee et al., 2008). Another evidence also acknowledged erk phosphorylation under bisphenol-A exposure (Watson et al., 2010).

1.1.2 Phthalates

Phthalates is an emergent topic of today. The current research nowadays, concentrates on finding protection from exposure to phthalates as well as defining mechanism by

which they can affect the organism. Phthalate is a widely used material. Cosmetics, pharmaceuticals, toys, toiletries, lotions all contain phthalates. Phthalates in non-genomic way affect the development, cell proliferation and gene expression. The study with rats that were exposed to the high doses of phthalates showed that phthalates are embryofetal toxins (Jobling et al., 1995). The female rats in this study have undergone spontaneous abortion and the litter size was diminished. This is arguably due to the decrease of plasma progesterone levels, which is possible controlled by non-genomic way of xenoestrogen (Jobling et al., 1995). Phthalates have common characteristics with estrogen, and it inhibits the acetylcholine receptors which are important in transmitting signaling in the brain (Liu and Chen, 2006). As well 2PX purinoceptors have been associated with the phthalate toxicity. The 2PX purinoceptors are widely distributed in the human system both in the central and peripheral nervous system, which are responsible for the olfactory, visual and sensory systems. The research performed on PC-12 cells has revealed that 2PX purinoceptors are getting blocked under phthalate toxicity. Phthalates induce ROS production. In a study of human granulocytes, ROS production was triggered when phthalates forced Ca^+ influx from the extracellular matrix into the cells (Palleschi et al., 2009). This brings awareness about how people can protect themselves from this gradual exposure. Newborns and pregnant women are mostly vulnerable to this kind of exposure. Many studies conducted have revealed that phthalates and bisphenol-A are mostly dangerous to fetuses and newborns.

1.2 ROS

Reactive oxygen species have different intracellular sources and have diverse physiological functions. The sources of ROS in the brain, are organelles and enzymes:

mitochondria, nitric oxide synthase, arachidonic acid metabolism, xanthine oxidase, monoamine oxidase and P450 enzymes (Schulz et al., 2000). These ROS species induce an effect on the proteins, lipids and DNA, by which they impose damage which then leads to neurodegeneration (Schulz et al., 2000) (Moskaug et al., 2005). Reactive oxygen species like H_2O_2 is one of the main reactive oxygen species that is responsible for alterations in the proteins, membranes, and DNA. Fenton reaction products are further generated when H_2O_2 reacts with Fe^{2+} (Then et al., 2009). These reactive molecules are further oxidized and then affect the proteins, DNA and other structures.

1.3 Oxidative stress.

Oxidative stress is the state where ROS levels are above a toxic threshold as cellular antioxidant defenses are deficient in the cells (Schulz et al., 2000). As well low levels of such intracellular antioxidant as glutathione hypothesized to lead cells into apoptosis. This has been shown in several studies, for example one study has shown that low levels of glutathione leads to the activation of neuronal 12-lipoxygenase (12-LOX) in both the cortical neurons and a neuronal cell line (Schulz et al., 2000). Oxidative stress occurs when the concentrations of the antioxidants are decreased (Schulz et al., 2000), and compound like nitric oxide, iron and especially reactive oxygen are generated (Moskaug et al., 2005). Oxidative stress is the first initiating effect in neurodegenerative diseases, where neuronal apoptosis is the main characteristic (Then et al., 2009). Parkinson's disease is believed to be caused from inhibition of oxidative phosphorylation, excitotoxicity and generation of ROS (Schulz et al 2000).

1.4 Deprivation and apoptosis

When PC-12 cell are deprived of serum, the cells initiate into apoptosis. Deprivation is a widely used model to study the ROS production in neurons (Satoh et al., 1996). Studying of ROS in the serum-free medium is also performed on the PC-12 cells which are easy to manipulate (Zhou and Zhu, 2000). ROS can generate both necrosis and apoptosis. Apoptosis happens at low concentrations of ROS, while necrosis is believed to evoke at the higher concentrations of ROS (Higuchi et al., 1998). The exact concept on where the ROS is generated is not yet known, but some evidence from earlier research suggest that mitochondrial respiratory chain can be one of the sites where ROS is generated (Higuchi et al., 1998).

1.5 MAP-kinase

Mitogen-activated protein kinase (MAPK) consists of 3 subfamilies: extracellular-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38MAPKs. All these molecular pathways constitute a very complex paradigm of pathways by which they infer regulation of cell signaling in the brain. MAP-kinase have earlier been reported to be involved in the deprivation and the toxicity by phthalates and bisphenol-A (Lee et al., 2007). MAP kinase is known to be involved in ROS production (Satoh et al., 2000, Xia et al., 1995).

-Erk

Extracellular-regulated kinases (ERKs) are involved in cell growth and cell differentiation and processes like mitosis and meiosis. There are many different Erk's

(ERK1, ERK2, ERK3, ERK4, ERK5) responsible for different processes of cell signaling (Fukunaga and Miyamoto, 1998).

-Jnk

C-Jun Terminal kinase is one of the MAP-kinases that is involved in the apoptotic cell death and control stress responses. There are three types of JNK classified til today (JNK1, JNK2, JNK3) (Kuan et al., 1999). C-Jun is involved in death as well as in survival and cell differentiation because it has a number of pleiotropic effects (Leppa et al., 2001).

-P38

P38 MAP-kinase is involved in apoptotic cell signaling pathway. It is also central in cytotoxicity and neurodegenerative processes (Gomez-Lazaro et al., 2007). There are four types of p38: p38 α , p38 β , p38 γ , and p38 δ . P38 MAPK increased activity is involved in oxidative stress and the control of stress responses (Then et al., 2009). High activity of p38 and its phosphorylation is linked to the accumulation of tau proteins, which lead to Alzheimer's disease (Then et al., 2009). Jnk and p38 MAP-kinases are central in the degeneration of neurons. High phosphorylation of both Jnk and p38 have been seen in the examination of the subjects that have Parkinson's disease (Chongthammakun et al., 2009). P38MAPK and erk are both involved in the neuronal differentiation (Morooka and Nishida, 1998).

1.6 Caspase-3

Caspases (cystein-aspartic proteases) are believed to be involved in cell death. When ROS is generated, these molecules affect the signaling pathways through a number of different caspases. Bcl-2 and Bax expression is found to be essential for the activation of the caspases in cell death (Then et al., 2009). Caspase-8 and caspase-9 are also thought to assist in the cell death signaling along with caspase-3 (Then et al., 2009). Caspase-3 is essential for normal brain development and signaling (Porter and Janicke, 1999). Caspase-3 protease is involved in apoptosis and necrosis induced by ROS (Higuchi et al., 1998). MAP kinase is known to regulate caspase-3 activity (Pearson et al., 2001, Sarker et al., 2000).

1.7 Glutathione an endogenous antioxidant

“ γ GT is a membraneous ectoenzyme that catalyzes the transfer of the γ -glutamyl moiety from GSH or a glutathione conjugate onto an acceptor molecule (Schulz et al., 2000). Ectoenzyme γ GT and its substrate – extracellular GSH work together to produce the dipeptide cysteinylglycine, which is further hydrolyzed to cysteine and glycine (Schulz et al., 2000). Cysteine and glycine are in turn the once responsible for the production of GSH.

GSH intracellular synthesis consists of two synthesizing enzymes that are ATP-dependent: γ -glutamylcysteine synthase (γ -GCS) and γ -glutathione synthetase (GSH) (Kondo et al., 1993). Gamma glutathione synthetase is a rate-limiting enzyme (Yao et al., 1995). γ -GCS is composed of two chains the heavy and the light chain. The reaction of γ -GCS is performed in a manner of feedback inhibition by GSH (Tsuboi, 1999). The heavy chain is a catalytic part of the enzyme, while the light chain carries regulatory actions (Yao et al., 1995). The decrease in the glutathione levels was connected with the reduction of the activity of AP-1 response element, which further gave decreased activity of γ -GCS (Yao et al., 1995). AP-1 transcription factor plays an important role in signal transduction in many types of cells (Liu et al., 2002). It is important for mitogenic signal transduction and is controlled by many growth factors. Jun and Fos proteins make up the AP-1 transcription factor. The regulatory region GCS is composed of two AP-1 transcription factors, nuclear factor- κ B (NF- κ B), and four antioxidant response elements (AREs) (Øverby et al., 2008). In the recent study, it was demonstrated that Nrf and Jun proteins bind to the antioxidant response elements and initiate their expression in response to xenobiotics and antioxidants (Jeyapaul and Jaiswal, 2000). It is believed that AP-1 is responsible for down regulation of γ -GCS promoter when α -estradiol is added to the serum-deprived PC-12 cells (Øverby et al., 2008) (Fallgren, Mathisen et al., 2007). Vitamins on the other hand did not down regulate γ -GCS promoter in the deprived PC-12 cells (Øverby et al., 2008). The

addition of the exogenous glutathione to the deprived PC-12 cells did not give any significant increase in the γ -GCS promoter (Øverby et al., 2008). This study gives interesting bases to further explore how deprivation together with α -estradiol would affect the glutathione levels in the PC-12 cells.

It was also found that the reduction of the mitochondrial GSH, not the cytosolic one, is responsible for the generation of ROS and inhibition of oxidative phosphorylation in PC-12 cells. Glutathione is homeostatically controlled and it is an essential molecule that is responsible for many different functions. Detoxification of xenobiotics, redox reactions and glutathionylation are all the main functions of this compound (Moskaug et al., 2005). Figure 1.1 shows a chemical structure of reduced glutathione and oxidized glutathione as a result of reaction.

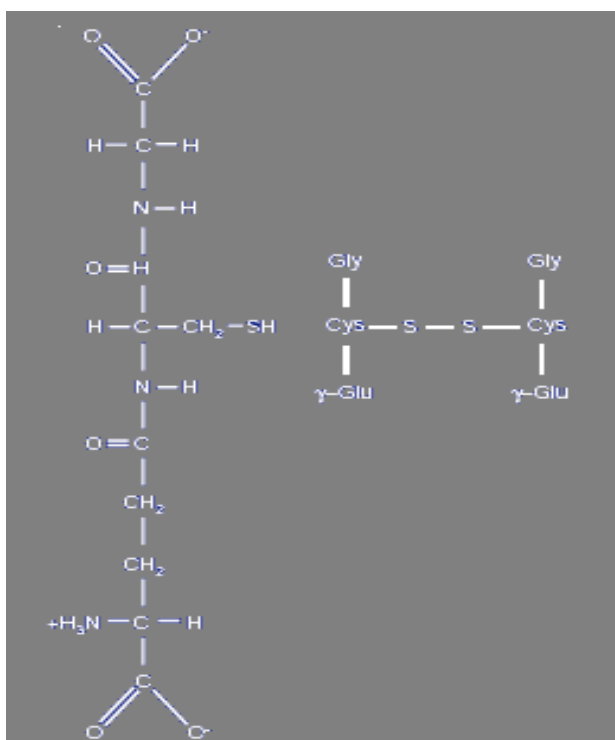


Fig. 1.1: Reduced glutathione (GSH) and oxidized glutathione (GSSG). The diagram depicts the two common forms of glutathione: the reduced glutathione to the left and the oxidized form to the right (Meister 1988).

Evidence towards activation of the LOX-pathway have been reported emphasizing that lipoxygenase-12 is an enzyme that gets activated when glutathione levels fall through nitric oxide pathways (Li et al., 1997). This in turn leads to the neuronal death when Ca^{+} influxes overrun the neuronal cells. Normal concentrations of the glutathione level not only protect cells but play an essential role in detoxification (Moskaug et al., 2005). Abnormal levels or depletion of glutathione levels is linked to carcinogenicity and evolvement of tumors (Moskaug et al., 2005). Many diseases are connected with oxidative stress, ROS and glutathione depletion. The slightest change in the level of oxidative stress would lead to outburst of ROS, depleting and using the GSH levels. Parkinson's disease, Alzheimer's disease, cardiovascular, chronic diseases, musculoskeletal and neurodegenerative diseases are consequences of the reduced (GSH) glutathione depletion. Many studies show that the reduced form of the glutathione is the one responsible for the vital processes in the cells (Fig.1.2). The study on subjects with chronic diseases, progression of tumors and with musculoskeletal disorders has been carried out against the control group. The study showed that the blood glutathione levels of the total glutathione have decreased in the patients compare to the control. This was due to the reduced GSH levels that dramatically decreased (Lang et al., 2000).

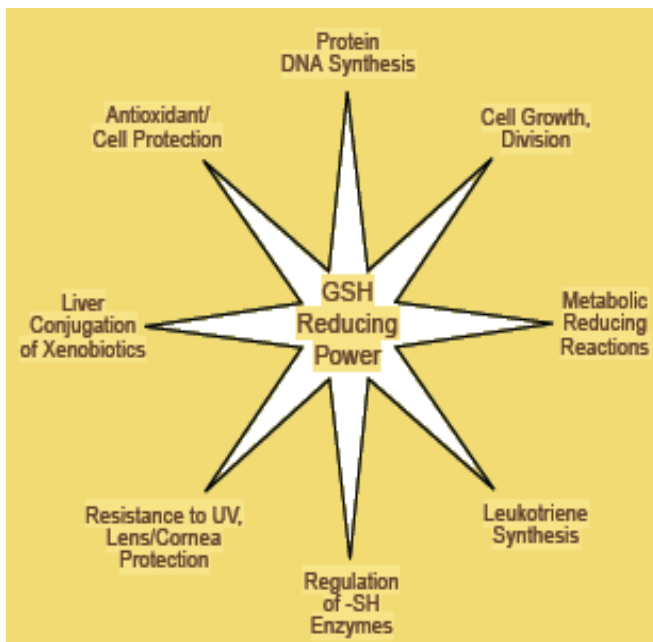


Figure 1.2: Cellular processes connected with the reduced glutathione. Many cellular processes are involved with the reduced glutathione. GSH is involved in the following: cell growth and division, protein and DNA synthesis, antioxidant and cell protection, liver conjugation of xenobiotics, resistance to UV and lens/cornea protection, regulation of $-SH$ enzymes, synthesis of leukotriene, metabolic reducing reactions. (Meister 1988)

Chronic diseases and longevity do depend on the amounts of reduced GSH in the cells of the organism. Age has been associated with decrease in reduced levels of glutathione (Lang et al., 2000). Many emphases have illustrated again and again that glutathione is one of the most essential molecules with very complex reactions. With the science advancing, there is a hope to find some therapeutic approach knowing the GSH's significance.

1.8 Antioxidants

Antioxidants in its nature are radical scavengers. Protective capabilities of vitamin E, specifically α -tocopherol (αT) many times have been registered in many studies. First

of all, Vitamin E is a neuroprotective antioxidant which helps to prevent apoptosis in neuronal cells (Then et al., 2009). Many studies of α -tocopherol have been implicated. Vitamin E is found to protect against neurodegenerative disease such as Alzheimer's disease, Down syndrome and others. It is believed that vitamin E adjusts signal transduction activity of redox-sensitive proteins like PKC- δ , PKB (Then et al., 2009). When treating hippocampal cells with α T before the exposure to the Fenton reaction particles, it was found that α T has protected the cells through the initiation of the gene expression (Then et al., 2009). The reduction in the activity of p38 MAP-kinase has been reported when human neuroblastoma cells were treated with vitamin E (Gomez-Lazaro et al., 2007). Vitamin E is also involved in the initiation of apoptosis in cancerous cells.

1.9 PC-12 cells cell line

The PC-12 cells are a pheochromocytoma cell line. It is used as a neural model to study cell signaling and apoptosis. The PC-12 cells grow and differentiate in the serum-medium, but when the serum is not present the cells undergo apoptosis. By itself, PC-12 cells is a useful model as both mitotic and post-mitotic differentiated cells can be used to study the developing neuron (Lindenboim et al., 1995). Nerve growth factor(NGF) is used to induce neurite formation and PC-12 cells become differentiated (Li et al., 1998). In current research undifferentiated PC-12 cells were used. This cell type is also used to determine erk and p38 function in survival and death respectively (Xia et al., 1995). The PC-12 cell model is a good model for studying ROS production (Zhou and Zhu, 2000, Xia et al., 1995).

1.10 Chicken cerebellar granule neurons

Chicken cerebellar granule neurons are a great and alternative model from PC-12 cells to study the real neurons. Rat's cerebellar granule neurons were the first model to study the real neurons (Gallo et al., 1982). Chicken cerebellar granule neurons is a recent model which allows to study excitotoxicity of neurons (Jacobs et al., 2006). The chicken cerebellar granule neurons do have a higher basal death than rat cerebellar granule neurons (Jacobs et al., 2006). Erk2 activation shows protection in chicken granule neurons as well as ROS production and caspase-3 activation is documented (Jacobs et al., 2006). Since the chicken cerebellar granule neurons undergo maturity, it is possible to study the toxicity of compounds at the different stages of the neuron development. This in turn suggests that earlier stages of chicken neurons development are representatives of earlier stages of the human fetus neuron development.

1.11 Aim of the study

- 1) To investigate the effect of deprivation, bisphenol-A and phthalates on glutathione levels.
- 2) To investigate if the exogenous addition of glutathione ester can substitute for the loss of endogenous glutathione during serum deprivation.
- 3) To study the effect of bisphenol-A and phthalates on the PC-12 cells and the mechanisms involved, such as: MAP kinase, caspase-3 activity and glutathione levels.
- 4) To examine the effect of bisphenol-A on the chicken cerebellar granule neurons, and examine if the mechanisms behind neuron toxicity differ from the PC-12 cell model.

2 Materials and Methods

2.1 Splitting of PC-12 cells

PC-12 cells are splitted on day 3 or day 4 when the whole bottom of the bottle of 175 cm² is filled with cells.

1. Old medium of 20ml is poured out.
2. 10ml of newly fresh PC-12 medium is added to the same bottle.
3. Hit slightly and shake the bottle on one side so that all the cells will detach from the bottom.
4. With the help of pipette move the medium up and down for all the cells to spread along equally in the medium.
5. See in the microscope, cells have to be detached from each other. If the cells are still in the clumps of several bunches use a glass heated pipette for separating the aggregates of cells.
6. 1.5 ml of this medium is used to make a new bottle of the PC-12 cells. 20ml of PC-12 medium is added to the 1.5 ml cell culture. Move bottle slightly so that the cells get distributed equally. Bottle is ready in the next 3-4 days for another splitting.

For splitting of the PC-12 cells in the dishes, the following formula is used:

$$X \text{ (ml cell suspension)} = \frac{\text{number of ml per dish} \times \text{number of dishes} \times \text{cell density of } 3.5 \times 10^4}{\text{cell density } 3.5 \times 10^4}$$

PC-12 cell culture medium:

Component	Amount
Dulbecoos MEM (DMEM)	500ml
Calf Serum	50ml
Horse Serum (HS)	25ml
Natrium pyruvate (100mM)	5ml
Penicillin- Streptomycine (5000IU/ml-5000UG/ml)	5ml

2.2 Deprivation of PC-12 cells

The cells are deprived on day two.

1. The dishes that are needed to be deprived are selected and the medium from the dishes is poured out.
2. Wash with 2ml serum-free medium (serum free medium recipe see below).

-
3. Wash dishes with 2ml of serum free medium, 2ml or 3 ml of serum free medium is added. Serum free medium for PC-12 cells:

Component	Quantity
Dulbecos MEM (DMEM)	500 ml
Sodium pyruvate (100 mM)	5 ml
Penicillin-Streptomycin (5000IU/ml-5000UG/ml)	5 ml

2.3 Treating PC-12 cells with antioxidants

Exposure concentrations:

Concentrations on cells	
Vitamin C	2 μ M
Vitamin E	10 μ M
17 α -estradiol	10 μ M
Glutathione	2.5 μ M and 5 μ M

2.4 Cell Death count for PC-12 cells

Colored blue cells: dead cells

White/transparent cells: live cells

1. First of all we take the 3ml dishes and pour out 2 ml of medium, so that there is only one ml left in the dish. Then we pipette out around 235 μ l of trypan blue and add to the dish.
2. This is done for all of the dishes. Then we set the dishes for incubation at 37°C with 5% CO₂ for 30 minutes.
3. After the dishes are incubated, we pore out the medium with trypan blue, and use microscope to investigate dead cells.

2.5 Making of Trypan Blue

Trypan Blue 2% Solution

Trypan Blue	1g
Distilled Water	50ml

Add trypan blue with water; mix the solution on the heat till the boiling point. Then cool down solution and filter. Trypan blue stored at the room temperature.

NaCl stock solution (1.8g/100ml):

NaCl	1.8g
Distilled water	100ml

Combine trypan blue solution and NaCl stock solution.

2.6 Bisphenol- A Concentrations

Bisphenol-A (C₁₅H₁₆O₂) concentrations:

100 µM, 200 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, 1M

Be aware: Bisphenol-A is irritant, use gloves and hood

Bisphenol-A and MEHP obtained from National Health Institute

2.7 Phthalate Concentrations

MEHP obtained from National Health Institute of Oslo.

Concentrations used: 100µM, 200 µM, 400 µM, 300 µM, 500 µM, 600 µM, 700 µM, 1M

2.8 Harvesting cells for glutathione measurement

Harvesting of the cells for glutathione measurement in 3 ways:

First kit- Cell Lysis Buffer: 235 µl of CLB for harvesting.

First kit-RIPA buffer for harvesting

Second kit - PBS for harvesting

2.9 Glutathione kit one (ApoGSH detection kit)

Procedure for the medium dishes:

1. Collect the dishes with different treatment and pour out all the rest of the media. Take and pour out 4 or 5 dishes at the time, so that they don't dry fast.
2. Wash dishes two times with cold PBS (keep PBS on ice)
3. Wash with PBS, remove the rests of PBS with 1ml pipette
4. Add 235 µl of cell lysis buffer in each dish and incubate for 10 minutes on ice
5. With the scrape harvest the cells into the eppendorff tubes. Scraping cells together is done into two directions perpendicular. Lean the dish with cells on the lock of the dish, so that cells concentrate in one region.
6. Place samples in eppendorf tubes and pipette out 60 µl for protein measurements. Mark eppendorf tubes, and store at -20°C.
7. The eppendorf tubes for glutathione centrifuge then at 13000 rpm at 4°C for 10 minutes.
8. Take then 96 well plate and mix 50 µl sample with 50 µl cell lysis buffer in a well.
9. 1 µl of MCB and 1 µl of GST to each well is added
10. Check for bobbles, if they persist, try to remove them with the needle.

11. Incubate the plate for approximately 2 hours at the 37°C

12. Measure the fluorescence

RIPA buffer tested with glutathione ApoGSH kit.

The scheme:

2.9.1 Cell Lysis Buffer vs RIPA Buffer in glutathione test measurement

		CLB			RIPA	
	<u>C1</u>	<u>D1</u>			<u>C2</u>	<u>D2</u>
1µl reagents	X1	X3		1µl reagents	X5	X6
0,5µl reagents	X2	X4		0,25µl reagents	X7	X8

X represents the glutathione measurement.

2.10 Glutathione kit two

Glutathione Assay Kit (Product Code CS0260) from Sigma. The kit measures the total glutathione (GSSG+GSH) through the deprotenizing of the biological sample.

Components of the kit:

Name	Components	Quantity
Assay Buffer 5x for Glutathione Product Code A 5103	500mM potassium phosphate, pH 7.0, containing 5 mM EDTA	30 ml
Glutathione Reductase Product Code G 3664	400 units per ml of glutathione reductase from bakers yeast in 3.6 M ammonium sulfate, pH 7.0, containing 0.1 mM dithiothreitol	20 units
Glutathione Reduced, Standard Product Code G 4544		0.3 mg

5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) Product Code S 2130		8 mg
5- Sulfosalicylic Acid Product Code N 6505		2.5 g
NADPH Product Code N 6505		25 mg
Dimethyl Sulfoxide (DMSO) Product Code D 8418		7.5 ml

Other equipment needed for fulfillment of this experiment:

- 96 well plate
- Plate reader
- Multichannel pipette

-
- Workout Victor Machine, Wallac 1420 Workstation is used here with 1420 multilabel counter

Samples harvested on 100µl of PBS, but before wash the treated dishes 2 times with PBS. Cells are scraped from the dish in two directions and are set in the eppendorff tubes and marked accordingly to each treatment.

2.10.1 Making of PBS:

Component	Final concentration	Quantity
KH_2PO_4	1.5 mM	0.24 g
KCl	2.7 mM	0.20 g
NaHPO_4	6.5 mM	1.44 g
NaCl	137.0 mM	8.01 g
Distilled water		1000 ml

Centrifuge at 600xg until packed pellet. Remove supernatant and the volume of the pellet is measured.

3 volumes of SSA of 5% Solution is needed to be added to one volume of the pellet.

Freeze and thaw the suspension. Repeat twice.

Leave for 5 minutes at 4°C.

Centrifuge the extract at 10000xg for 10 minutes.

Measure volume of supernatant and use this as the original sample volume in the further calculation of glutathione.

If the assay cannot be performed immediately (within 2 hours), then the extract may be stored at -70°C.

Stock solutions and working solutions:

Stock Solutions:

- DTNB Stock Solution (1.5mg/ml): dissolve the contents of the 8 mg of DTNB (Product Code D 8418) to make a 1.5 mg/ml solution. The aliquots are made, each of 228 µl. This stock solution is to be stored at -20°C for at least 3 month
- NADPH Stock Solution (40 mg/ml) is made by dissolving the NADPH (25 mg) in 0.625 ml of water to give a 40mg/ml solution. This stock solution should be further stored at -20 °C for at least 6 month.
- 5% 5- Sulfosalicylic Acid (SSA) Solution is made by dissolving 5-sulfosalicylic acid (2.5g) in 50 ml of water. This solution should be dissolved very well and kept at the 4 °C.
- The last stock solution to be made is Glutathione (GSH) Standard Stock Solution (10 mM). To achieve this we have to dissolve Glutathione Reduced, Standard in 0.1 ml of water. The solution is then stored at -20°C for at least 3 month.

Working Solutions:

- 1x Assay Buffer (12ml) is made by diluting 2.4 ml of Assay Buffer 5x (Product Code A 5103) five-fold by addition of 9.6 ml of water.
- Enzyme solution (6 units/ml, 0.25ml) by diluting 3.8 μ l with 1x Assay Buffer.
- NADPH Solution (0.16 mg/ml, 2.5 ml) is made by adding 10 μ l of NADPH Stock Solution (40mg/ml) to 2.5 ml of 1x Assay Buffer.
- Working Mixture : by adding 228 μ l of the Enzyme solution with 228 μ l of DTNB Stock solution and 8ml of 1x Assay Buffer.
- The last solution is Glutathione Standard Solutions, made by diluting an aliquot of the Glutathione Standard Stock solution (10mM) 200-fold to 50 μ M with the 5% 5- Sulfosalicyclic Acid Solution (SSA).

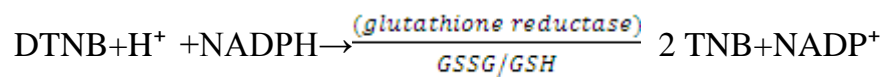
2.10.2 Glutathione Standard Solutions by serial dilutions is shown below in the table:

Well number	1	2	3	4	5
GSH Concentration (μM)	50	25	12.5	6.25	3.125
GSH Solution (μl)	50	25 from well 1	25 from well 2	25 from well 3	25 from well 4
5% SSA	None	25	25	25	25
Nmoles GSH in a 10μl sample	0.5	0.25	0.125	0.0625	0.0312

The reaction scheme:

1. $2\text{GSH} + \text{DTNB} \rightarrow \text{GSSG} + 2\text{TNB}$
2. $\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow (\text{glutathione reductase catalyzes the reaction}) 2\text{GSH} + \text{NADP}^+$

Combining the two reactions:



Kinetic assay is used to measure GSH. Reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to TNB and the GSSG is formed, then recycled by glutathione reductase and NADPH. TNB is the product that gives yellow color, and the glutathione is measured spectrometrically at 412 nm.

Machine used to measure the glutathione and register the kinetic energy: the Wallac 1420 Workstation.

Reaction scheme:

Sample measured	Sample volume	5%SSA	Working Mixture	NADPH(0.16mg/ml)
Reagent Blank	--	10 μ l	150 μ l	50 μ l
Standard curve (various dilutions)	10 μ l	---	150 μ l	50 μ l
Unkown sample	x μ l	10-x	150 μ l	50 μ l

The first two wells include 10 μ l of the 5% 5-Sulfosalicylic Acid Solution as a reagent blank.

All the wells contain 150 μ l of the Working Mixture. Make sure mixture is mixed well.

The incubation period is 5 minutes at the room temperature.

50 μ l of the diluted NADPH Solution is added with a multichannel pipette and the plate reader is used to gather the data through absorbance.

2.11 Protein measurement

For the measurement of the protein: Pierce BCA (bicinchoninic acid) Protein Assay Kit. This purple color in the reaction is a product of the chelation of the two molecules of BCA with one cuprous ion.

Kit Components:

BCA Reagent A (Product No. 23225) or (Product No. 23227)	contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide	1000ml or 500 ml depending on the product
BCA Reagent B	Containing 4% cupric sulfate	25 ml
Albumin Standard Ampoules	10x1ml ampoules, containing bovine serum albumin (BSA) at 2.0mg/ml in 0.9% saline and 0.05% sodium azide	2mg/ml

All the kit components are stored at the room temperature.

Spectrophotometer should be set to 562 nm when measuring the protein.

2.11.1 Solutions for protein measurement:

Working Reagent (WR)		Standard solution	
BCA assay reagent A	50 parts	Bovine serum albumin (2mg/ml)	50µl
BCA assay reagent B	1 part	PBS	150µl

2.11.2 Application of the standard:

	1	2	3
A	0	10µl	Sample 1
B	0	10µl	Sample 2
C	1,2µl	20µl	Sample 2
D	1,2µl	20µl	Sample 2
E	2,5µl	40µl	Sample 3
F	2,5µl	40µl	Sample 3
G	5µl	Sample 1	Sample 3
H	5µl	Sample1	

200µl of Working Reagent is added to all the wells that are used on the 96-well plate.

The first wells are for standard application.

10µl of the solution in which sample is harvested and is added to the wells together with the standard solution.

Pack plate into aluminum foil.

The incubation period is from 30 minutes up to 2 hours. Be aware to check for bubbles formation. This can interfere with the protein reader.

3. Results

3.1 Glutathione levels in the PC-12

Since glutathione is important for cellular protection, it was interesting to investigate glutathione level of PC-12 serum-deprived cells along with vitamin C and E, and α -estradiol. In previous work by Gunn Rebecca Øverby, it was shown that γ -GCS promoter activity falls in deprived PC-12 cells along with addition of the α -estradiol. Vitamins have been identified as mediators for increase in the glutathione level (Then et al., 2009). Both bisphenol-A and phthalates have been also reported earlier to be the environmental toxicants which increase ROS production (Palleschi et al., 2009, Ooe et al., 2005). The study therefore concentrates on glutathione level under deprivation (see 3.1.1) and under exposure of bisphenol-A (see 3.1.2) and phthalates (see 3.1.3). The experiment was done at the two time point, the 24 hour and 48 hour point.

3.1.1 Glutathione Level in control and Deprived PC-12 cells

A Glutathione level in PC-12 cells with serum was compared to PC-12 cells in the serum-free medium (deprived) (Fig. 3.1). Glutathione level in the control was much higher than the deprived.

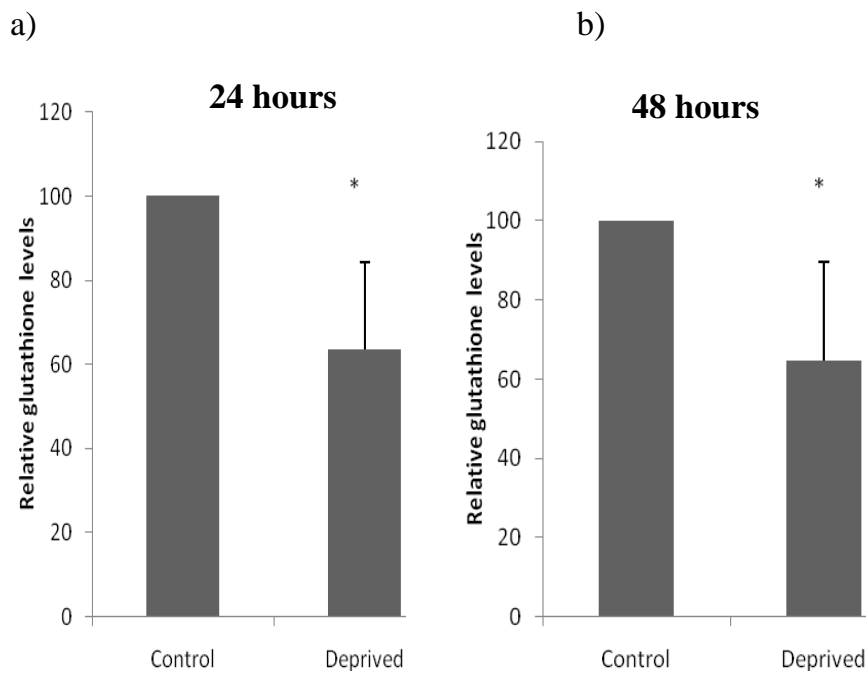


Figure 3.1: Glutathione levels in deprived and non-deprived PC-12 cells. The dishes of PC-12 cells were cultured for 24 hours (a) or 48 hours (b). Deprived dishes did not contain serum. The dishes were splitted on day one. On day two dishes were deprived. Measurement of glutathione was performed and glutathione levels were corrected for protein. Bars represent mean \pm SD.

- a) The differences in the mean values among the treatment groups at 24 hours is greater than would be expected by chance; there is a statistically significant difference ($P = <0,001$), $n=7$ independent experiments, two-sample t-test.
- b) The differences in the mean values among the treatment groups at 48 hours are greater than would be expected by chance; there is a statistically significant difference ($P = <0,001$), $n=11$ independent experiments, two-sample t-test..

3.1.2. Glutathione levels in Control with Antioxidants

Different antioxidants have different effect on the glutathione levels (Behl, 2000, Wang et al., 2006). Here it was investigated what happens when vitamins (vitamin C and E) and α -estradiol were added. The levels of glutathione did not show significant change compare to the control (Fig. 3.2).

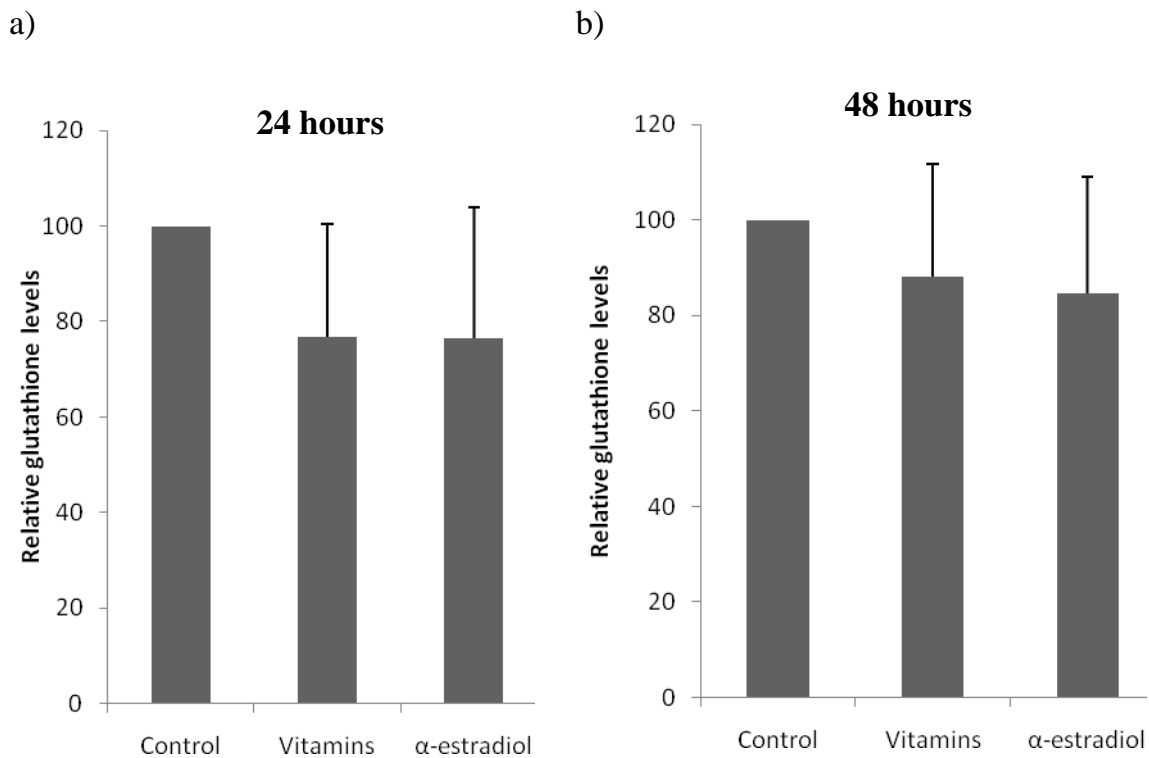


Figure 3.2: Glutathione levels in control PC-12 cells versus PC-12 cells treated with antioxidants. PC-12 cells were splitted on day one. On day two dishes were treated with antioxidant. Dishes were harvested for glutathione after 24 hours (a) after treatment and 48 hours (b) after treatment. Glutathione measurement was performed, and glutathione levels were corrected for protein. Bars represent mean \pm SD (standard deviation).

a) Statistical test shows that differences in the median values among the treatment groups at 24 hours are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=7$ independent experiments, one way Anova.

b) The differences in the median values among the treatment groups at 48 hours are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=10$ independent experiments, one way Anova.

3.1.3 Glutathione levels in Deprived PC-12 cells with antioxidants.

Since deprivation induces ROS, it was interesting to see if antioxidants would give any protection to glutathione level in PC-12 cells. Glutathione levels will show if ROS is abundant and if glutathione is used. Results showed (Fig. 3.3 a) that glutathione levels at 24 hours between the treatments did not show any major significant changes between the treatments. At 48 hours vitamins had a tendency of a higher glutathione level then the control (Fig. 3.3 b). α -estradiol on the other hand was significant. Reduction of glutathione level was detected.

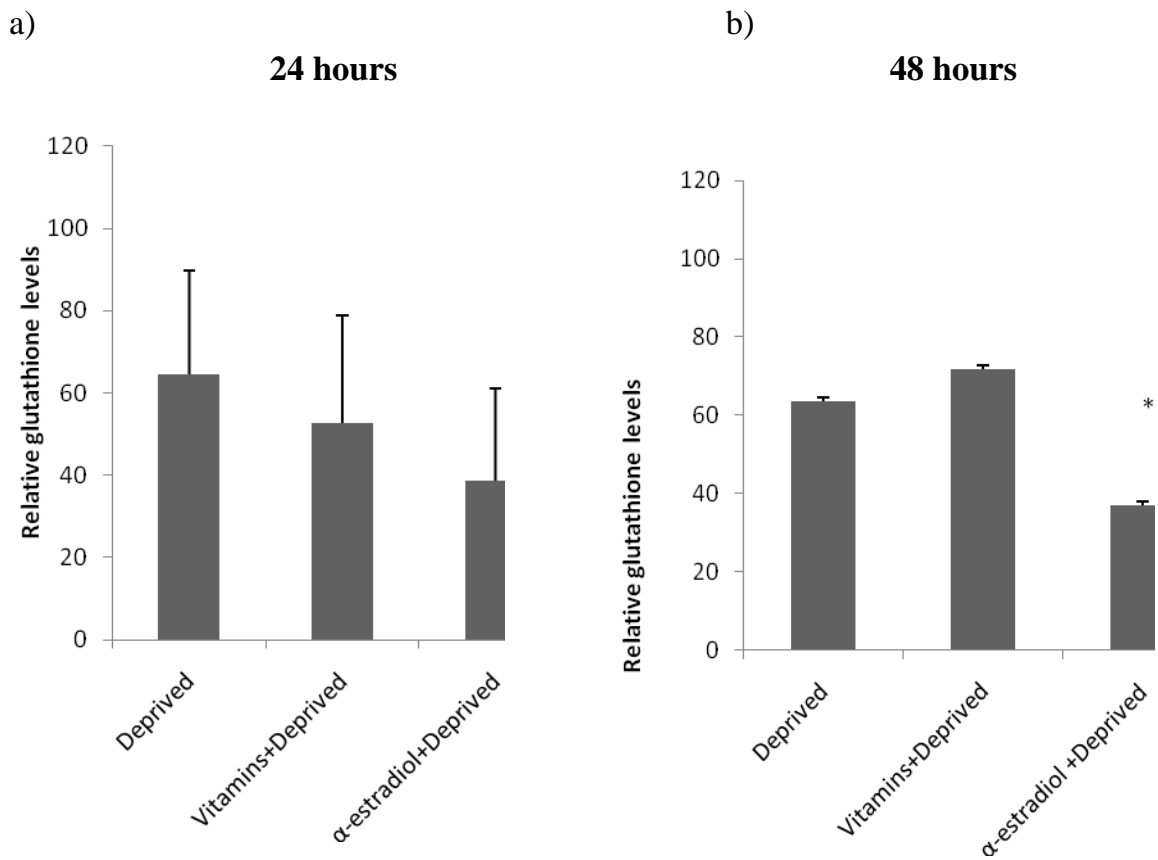


Figure 3.3: Glutathione levels in the deprived PC-12 cells with antioxidants. PC-12 cells were splitted on day one. On day two dishes were treated with antioxidants. Dishes were harvested for glutathione measurement after 24 hours (a) and others after 48 hours (b) after treatment. Glutathione measurement was performed, and glutathione levels were corrected for protein. Bars represent mean \pm SD (standard deviation).

a) The differences in the mean values among the treatment groups (deprived and deprived with α -estradiol) at 24 hours are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P = 0,073$), $n=7$ independent experiments, one way Anova.

b) The differences in the mean values among the treatment groups at 48 hours are greater than would be expected by chance; there is a statistically significant difference ($P = 0,012$). The significant treatment with α -estardiol is marked with the star in the (Fig. 3.3b.), $n=8$ independent experiments, Dunnett's method.

3.1.4 Glutathione levels after addition of exogenous glutathione control PC-12 cells

Exogenous glutathione was added to see if the glutathione levels in the serum-free medium would increase and therefore substitute for the endogenous glutathione (Fig. 3.4; 3.5)

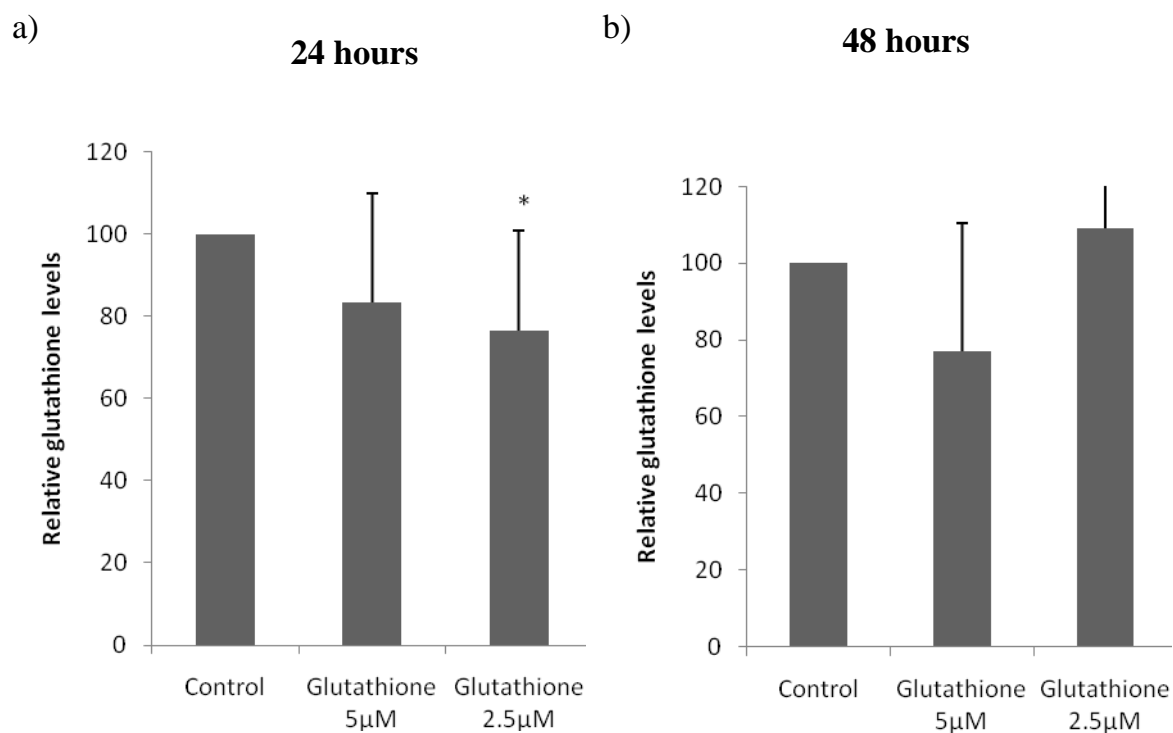


Figure 3.4: Glutathione levels after administration of glutathione in the control. Additional glutathione was administrated at two different concentrations to the PC-12 dishes. These concentrations of glutathione were 5 μM and 2.5 μM. PC-12 cells were splitted on day one. Dishes were treated on day two. PC-12 dishes were harvested for glutathione measurement after 24 hours (a) and other dishes after 48 hours (b) after the treatment. Glutathione measurement was performed, and glutathione levels were corrected for protein. Bars represent mean ± SD (standard deviation).

- a) The differences in the median values among the treatment groups at 24 hours are greater than would be expected by chance; there is a statistically significant difference ($P = 0,047$), ($P < 0.05$), where $n=5$ independent experiments, Dunnett's method. After the statistical test it is possible to see that the treatment with glutathione 2.5 μM is significant
- b) The differences in the median values among the treatment groups at 48 hours are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=7$ independent experiments, one way Anova.

3.1.5 Glutathione levels in PC-12 cells after administration of exogenous glutathione in serum-free medium.

It was interesting to see if the levels of glutathione increase after addition of extra glutathione after deprivation. No significant changes in the glutathione have been detected (Fig. 3.5; 3.6)

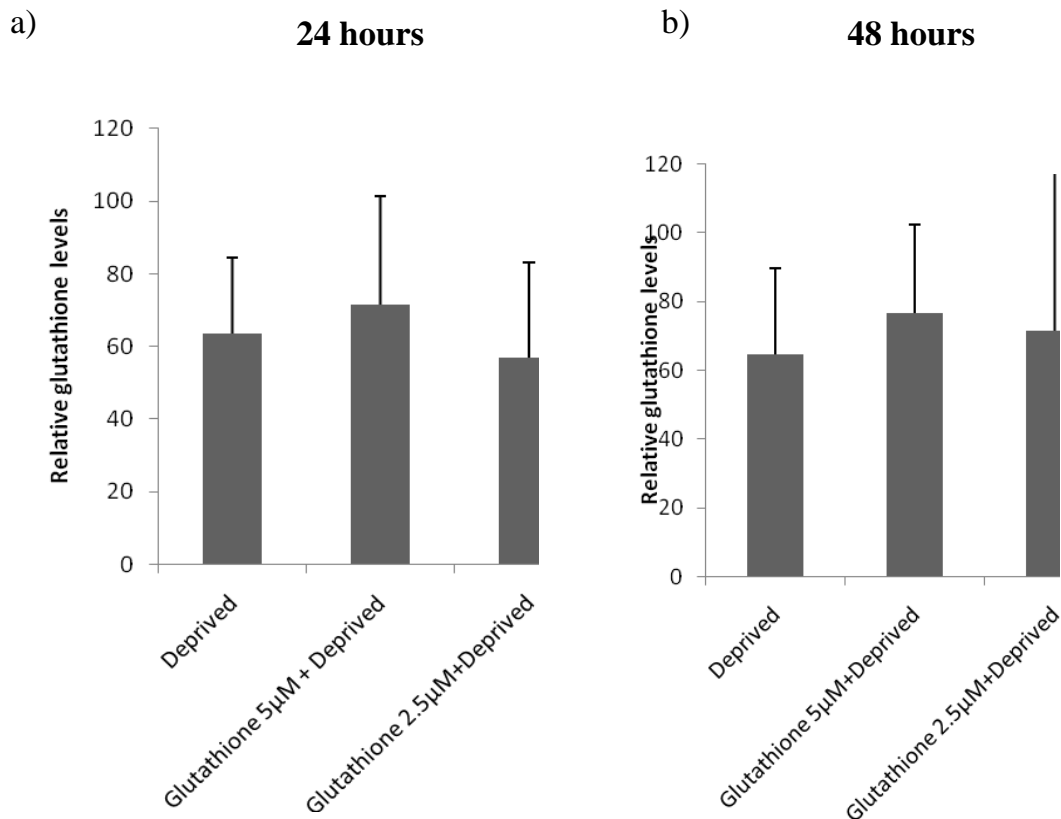


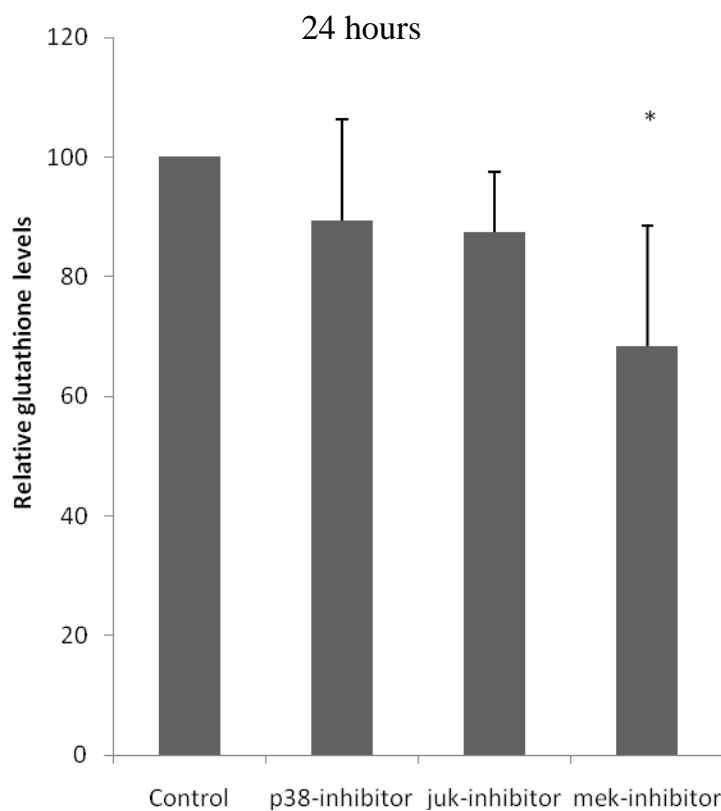
Figure 3.5: Glutathione levels after administration of additional glutathione in the deprived PC-12 cells. PC-12 cells were splitted on day one .Dishes were treated on day two. PC-12 dishes were harvested after 24 hours (a) and other dishes after 48 hours (b) after treatment. Glutathione measurement was performed, and glutathione levels were corrected for protein .Bars represent mean \pm SD.

- a) The differences in the mean values among the treatment groups at 24 hours are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=5$ independent experiments, one way Anova.
- b) The differences in the median values among the treatment groups at 48 hours are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=7$ independent experiments, one way Anova.

3.1.6. Mechanisms behind the regulation of glutathione levels in control PC-12 cells

It is known that MAP- kinase (MAPK) is involved in regulation of γ -GCS promoter (Xia et al., 1995). Therefore it was chosen to concentrate on the MAP kinase to investigate if it is involved in the regulation of the glutathione levels.

Results showed that at 24 hours (Fig. 3.6a) mek-inhibitor was significant. There was a definite increase in glutathione levels. Other treatments did not show any significant change from the control at 24 hours. At 48 hours (Fig.3.6b) not only treatment with mek-inhibitor was significant, but treatment with p38-inhibitor became significant too.



a)

b)

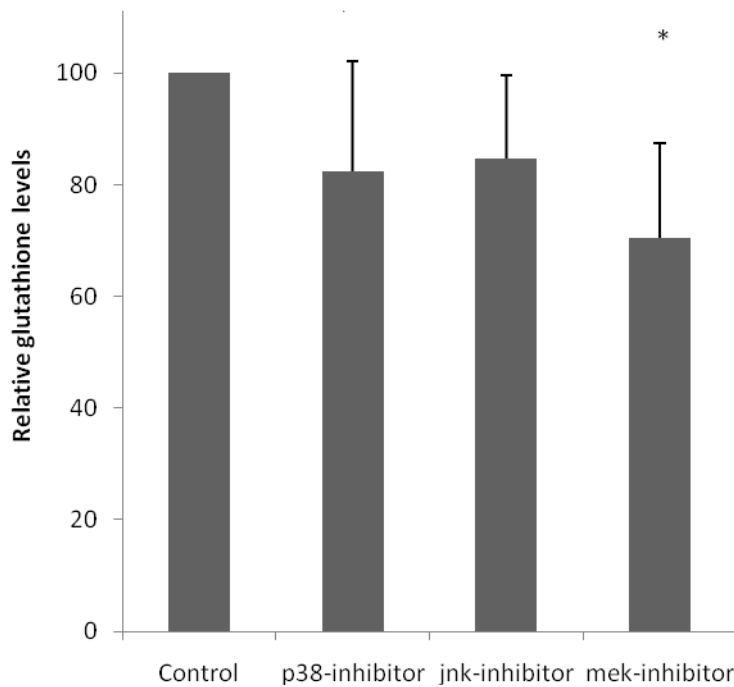
48 hours

Figure 3.6: Inhibiting of p38, jnk and mek pathway in non-deprived PC-12 cells. PC-12 cells were splitted on day one. Dishes were treated with inhibitors on day two. PC-12 dishes were harvested after 24 hours (a) and other dishes after 48 hours (b) after treatment. Glutathione measurement was performed, and glutathione levels were corrected for the protein. The bars indicate mean \pm SD.

- a) The differences in the median values among the treatment groups at 24 hours are greater than would be expected by chance; mek-inhibitor treatment is statistically significant ($P < 0.05$), where $n = 4$ independent experiments, Dunnett's method.
- b) The differences in the median values among the treatment groups at 24 hours are greater than would be expected by chance; there is a statistically significant difference: treatments with p38-inhibitor and treatment with mek-inhibitor are significant. P is less than 0.05, where $n = 5$ independent experiments, Dunnett's method.

3.1.7 Mechanisms behind regulation of glutathione levels in deprived PC-12 cells

Since MAP-kinase is involved in the cell death pathway it was interesting to investigate the glutathione levels after inhibition. This is done on the basis that ROS is produced at deprivation, as mentioned earlier. Therefore it was motivating to find out which pathway is involved in ROS inducement, as well as in utilization and production of endogenous glutathione. Results showed (Fig. 3.7) that there is no significant difference between all of the treatments for both time points. The tendency of decrease in glutathione levels when jnk-inhibitor is used can be noticed.

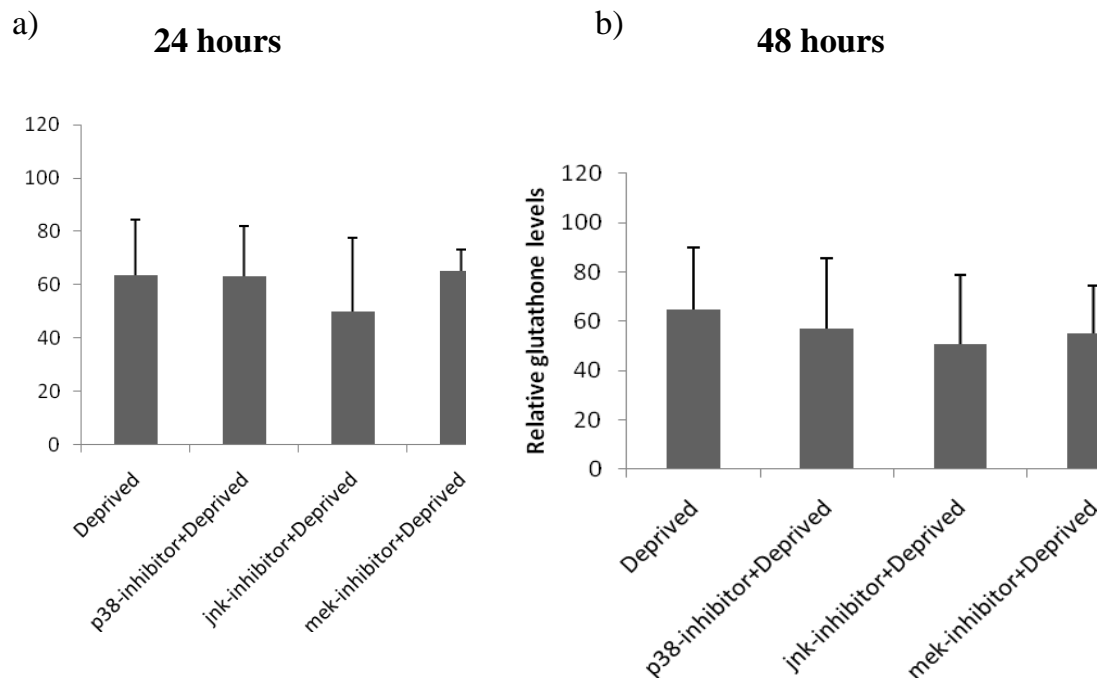


Figure 3.7: Inhibiting of p38, jnk and mek in deprived PC-12 cells. PC-12 cells were splitted on day one. Dishes were treated with inhibitors and deprived on day two. PC-12 dishes were harvested after 24 hours (a) and other dishes after 48 hours (b) after treatment. Glutathione measurement was performed, and glutathione levels were corrected for the protein. The bars indicate mean \pm SD. a)The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P = 0,060$), ($P > 0.05$), where $n = 4$ independent experiment, one way Anova. b)The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n = 5$ independent experiment, one way Anova.

3.1.8 Mechanism behind the fall of glutathione levels in PC-12 cells when α - estradiol is added

It was interesting to find the inhibitor had a tendency to stop the downfall of glutathione when we add α -estradiol to deprived cells (Fig. 3.3b). The results were the following (Fig.3.8;3.9): in control PC-12 cells the treatment with p38-inhibitor together with α -estradiol for 48 hours were not significant (Fig 3.8). The deprivation dishes could show if glutathione levels change. The decrease from the treatment with deprivation and just α -estradiol stopped having a decreased glutathione levels when p38-inhibitor was added (Fig. 3.9). When p38 pathway is blocked in the treatment with α -estradiol in deprivation, glutathione levels do not fall.

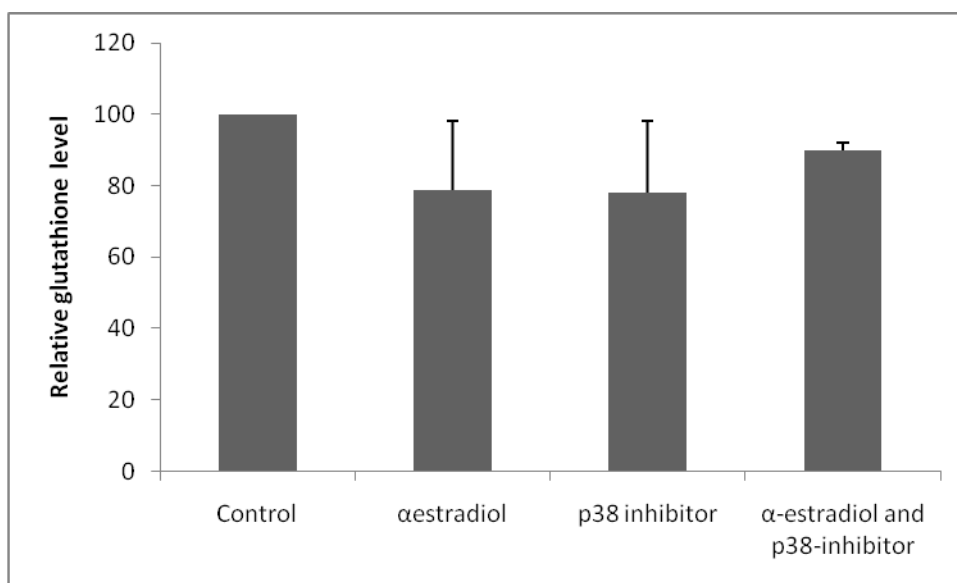


Figure 3.8: Inhibiting of p38 with addition of α -estradiol versus non-deprived PC-12 cells. PC-12 cells were splitted on day one. Dishes were treated with inhibitors and deprived on day two. PC-12 dishes were harvested after 48 hours after treatment. Glutathione measurement was performed, and glutathione levels were corrected for the protein. The bars indicate mean \pm SD.

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n = 4$ independent experiments, Dunnett's method.

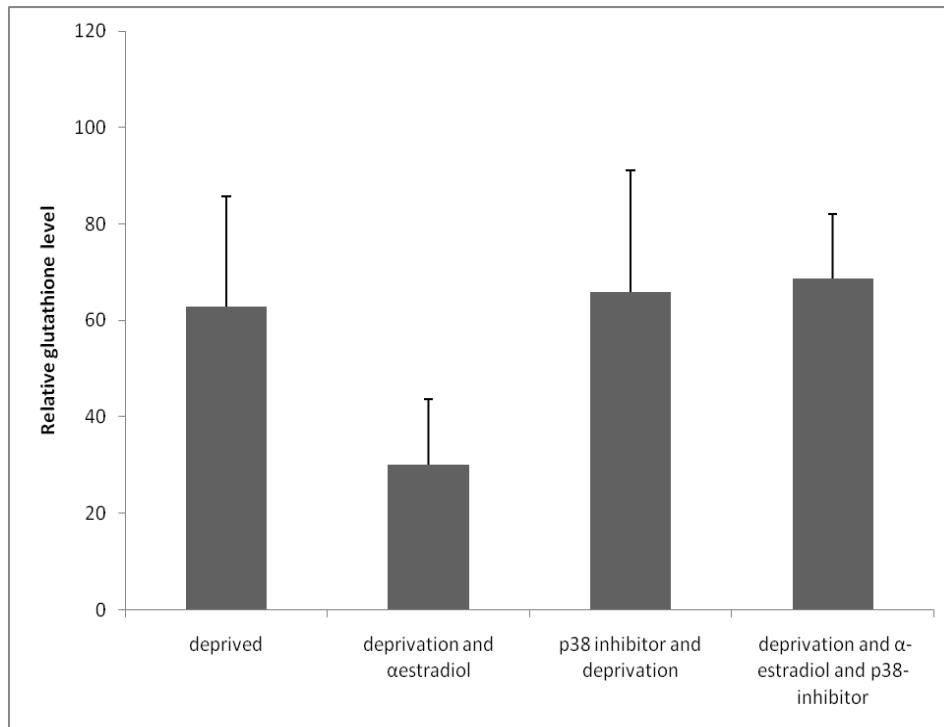


Figure 3.9: Inhibiting of p38 together with addition of α -estradiol in the deprived PC-12 cells. PC-12 cells were splitted on day one. Dishes were treated with inhibitors and deprived on day two. PC-12 dishes were harvested after treatment. Glutathione measurement was performed, and glutathione levels were corrected for the protein. The bars indicate mean \pm SD. The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference, P is larger then 0.05, $n = 4$ independent experiments, Dunnett's method.

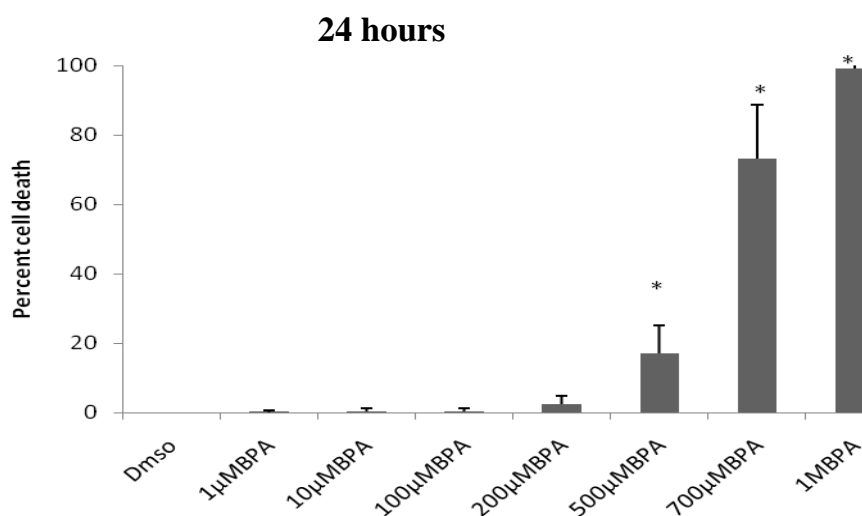
3.2 Bisphenol-A in PC-12 cells

In this part the effect of Bisphenol-A on PC-12 cells was studied. It was reported earlier that bisphenol-A causes ROS production (Ooe et al., 2005). At the same time MAP-kinase according to literature was shown to be involved (Zsarnovszky et al., 2005). Therefore it was chosen to inhibit p38, jnk and erk pathways. The effect of vitamins, p38-inhibitor, jnk-inhibitor and mek-inhibitor on PC-12 cells after bisphenol-A exposure was of an interest.

3.2.1 Dose-response curve of bisphenol-A in PC-12 cells

First, the dose-response data was obtained. Graph at the 24 hour point is shown in Fig. 3.10a. The results showed that cell death became apparent at 200 μ M of bisphenol-A administration. 1M bisphenol-A gave a total death of 100 percent. The dose-response increased greatly above 500 μ M bisphenol-A concentration. 200 μ M concentration was chosen on the basis of these results for further experiments. Figure 3.10 demonstrates dose-response at 24 hours (a) and at 48 hours(b) after exposure to bisphenol-A.

a)



b)

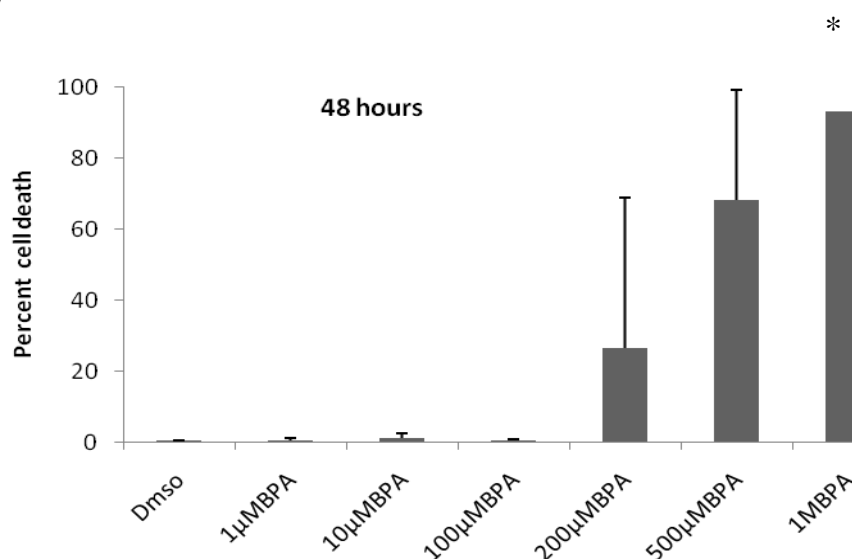


Figure 3.10: Dose-response for bisphenol-A in PC-12 cells. The PC-12 cells were splitted and plated on the dishes. The dishes were treated next day with different concentrations of bisphenol-A. Bisphenol-A concentrations were made by dissolving the compound in DMSO. After 24 hours and 48 hours of treatment dishes were treated with trypan blue for cell death counting. Bars here represent mean \pm SD.

a) Treatment with 500µM BPA, 700µM BPA, and 1M BPA are significant, where $n = 9$ independent experiments, Dunnett's method.

b) Treatment of 1M BPA is significant, where $n = 5$ independent experiments, Dunnett's method.

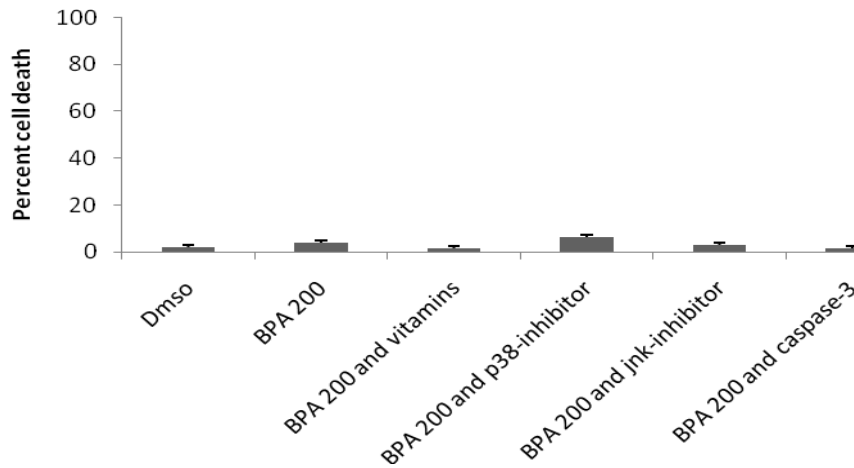
3.2.2 Mechanisms behind bisphenol-A toxicity in PC-12 cells

It is reported that MAP-kinase is involved in the cell death pathway after the administration of bisphenol-A (Tanabe et al., 2006).

Caspase-3 involvement in cell death pathway after bisphenol-A exposure is also reported in many types of cells (Iida et al., 2003, Terasaka et al., 2005).

The results did not show any significance (Fig. 3.11), but the trend that vitamins are involved in protection has been hypothesized. Jnk was also earlier reported to be involved in the programmed cell death pathway under bisphenol-A exposure (Lee et al., 2008). Blocking the jnk, did not decrease cell death.

a)

24 hours

b)

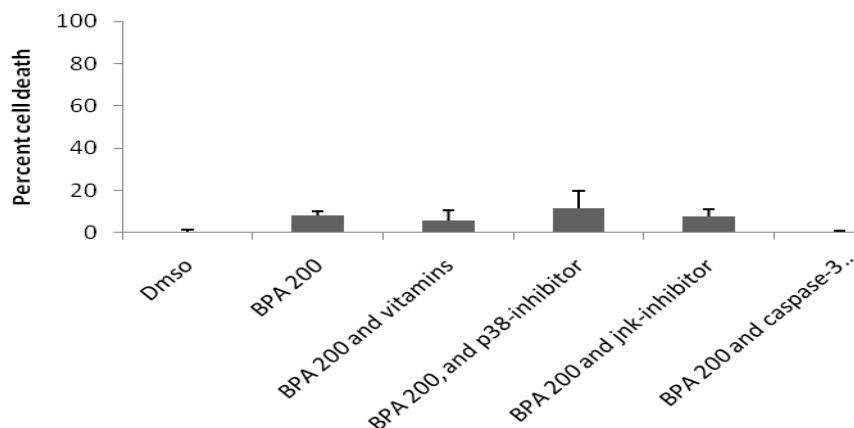
48 hours

Figure 3.11: Mechanisms involved in cell death at the exposure to bisphenol-A in PC 12 cells. The concentration of 200 μ M of bisphenol-A was chosen to investigate the toxicity along with different inhibitors. The PC-12 cells were splitted and plated on the dishes. Next day the dishes were treated. After 24 hours and 48 hours of treatment dishes were treated in trypan blue for cell death counting. Bars here represent the \pm SD. a) The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P = 0,060$), where $n = 6$ independent experiments, one way Anova. b) The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P = 0,198$), where $n = 7$ independent experiments, one way Anova.

3.2.3 Glutathione levels under exposure to bisphenol-A in PC-12 cells

Since cell death was measured in the PC-12 cells after bisphenol-A was added, it was interesting to look at the glutathione levels. A low concentration of 100 μ M was chosen and a higher concentration of 500 μ M was chosen. Results show that there are no significant differences in glutathione levels (Fig. 3.12)

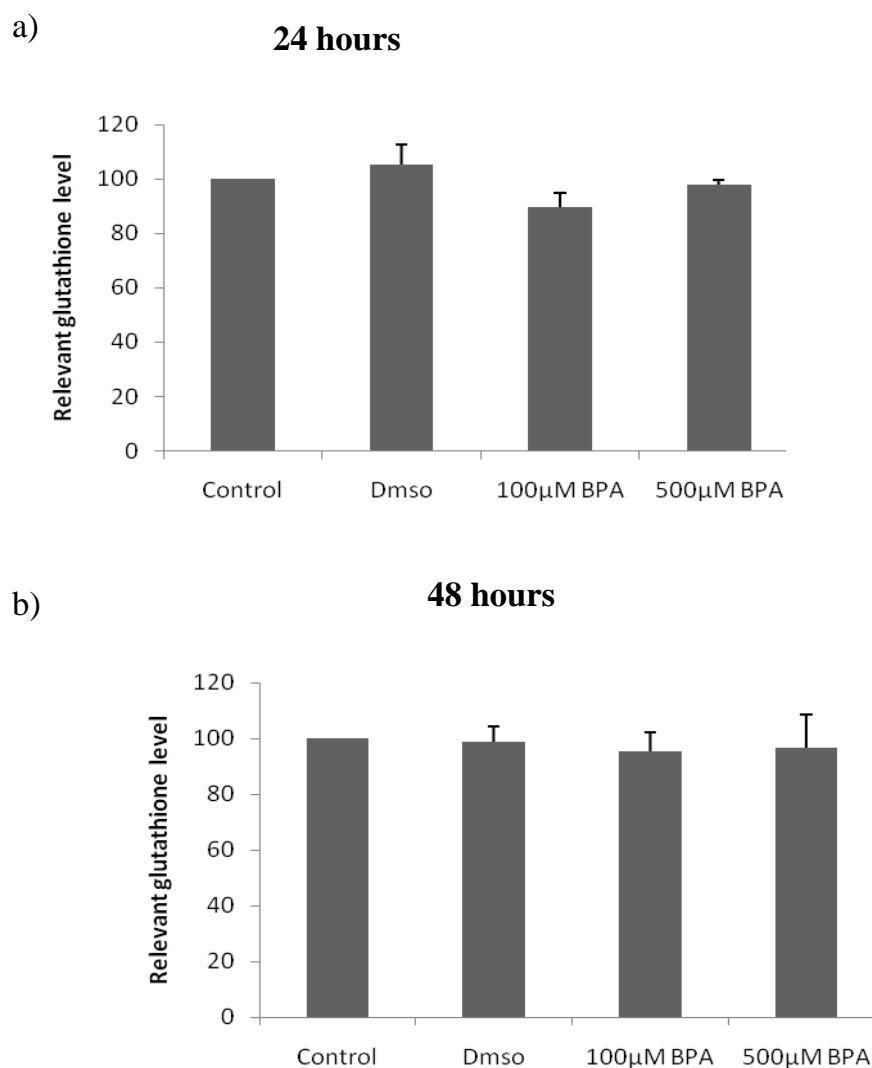


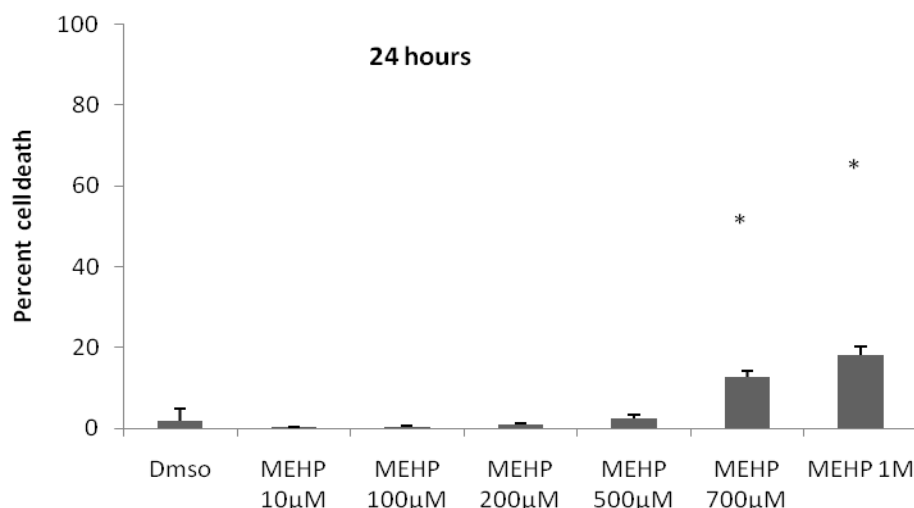
Figure 3.12: Glutathione levels at two concentrations of bisphenol-A in PC-12. PC-12 cells were splitted on day one. On day two dishes were treated with antioxidants. Dishes were harvested for glutathione measurement after 24 hours (a) and others after 48 hours (b) after treatment. Glutathione measurement was performed, and glutathione levels were corrected for protein. a) Here the data is represented from two dishes of two independent experiments with bars representing median, Mann-Whitney test. There is no significance between the treatments. b) No significance between the treatments. Bars represent mean \pm SD, where $n=4$ independent experiments, one way Anova.

3.3 Phthalate in PC-12 cells

3.3.1 Dose-response in PC-12 cells after phthalate exposure

It is known that phthalates produce reactive oxygen species in the cells (Palleschi et al., 2009), and therefore it was examined how concentrations of phthalate (MEHP) affect the cell death. MEHP gave significance above 500 μ M after 24 hours of treatment (Fig. 3.13a). After 48 hours of treatment with MEHP (Fig.3.13b), MEHP gave significance at 1M concentration.

a)



b)

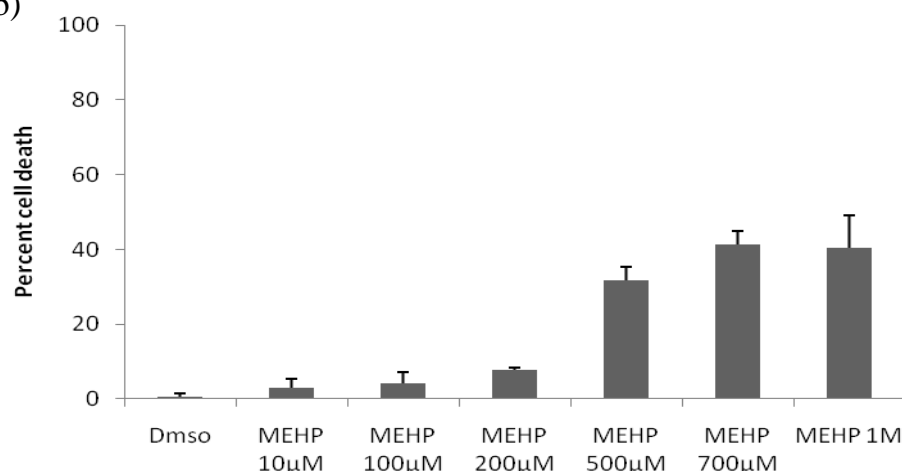


Figure 3.13: The dose-response curve after exposure to different concentrations of phthalates (MEHP) in PC-12 cells. The PC-12 cells were splitted and plated on the dishes. The dishes next day were treated with different concentrations of phthalates. Phthalate (MEHP) concentrations were made by dissolving the compound in the DMSO. After 24 hours and 48 hours of treatment dishes were treated with trypan blue for cell death counting. Bars here represent mean \pm SD.

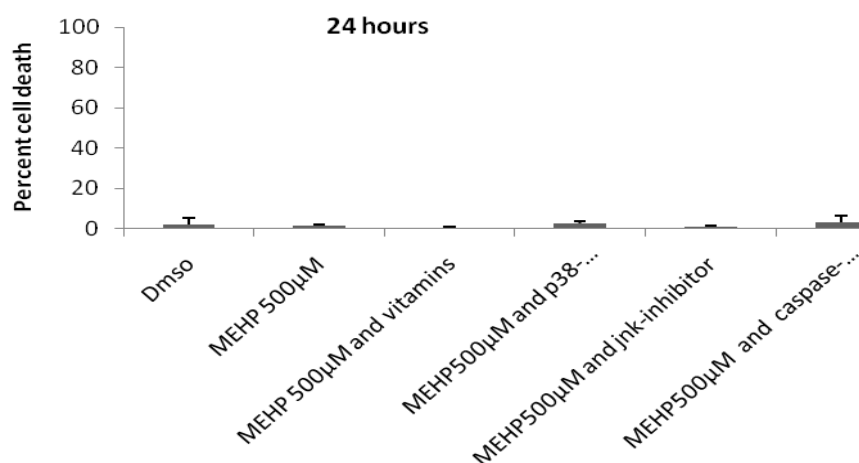
a) Treatments: 500µM BPA, 700µM BPA, and 1M BPA are significant, where $n=9$ independent experiments, Dunnett's method.

b) Treatment of 1M BPA is significant, where $n=5$ independent experiments, Dunnett's method.

3.3.2 Mechanisms behind phthalate toxicity in PC-12 cells

Since dose-response have been measured, it was decided to use 500 μ M concentration for phthalate (MEHP) to check for the mechanisms behind the phthalate toxicity. Since MAP-kinase is reported to be involved in the phthalate toxicity, MAP kinase-inhibitors, and other inhibitors such as ROS scavenger- vitamins, and caspase-3 were chosen for experiment. The results showed no significance (Fig. 3.14).

a)



b)

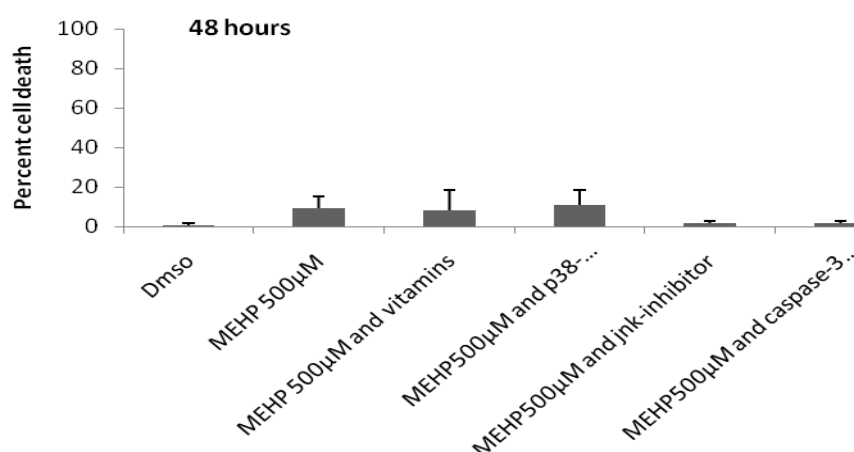
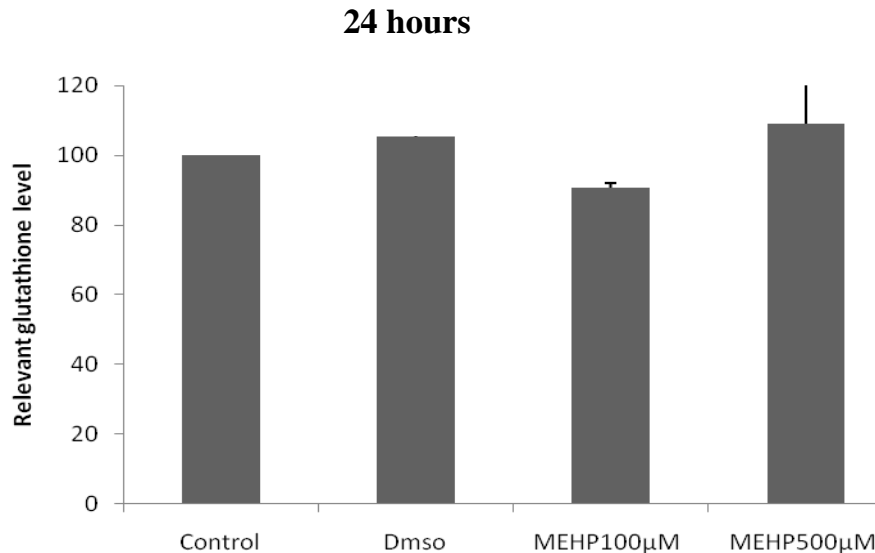


Figure 3.14: Mechanisms behind the cell death after phthalate exposure in PC-12 cells. The chosen concentration of 500µM of MEHP was used to investigate the toxicity along with different inhibitors. The PC-12 cells were splitted and plated on the dishes. Nest day the dishes were treated with different concentrations of MEHP. Phthalate (MEHP) concentrations were made by dissolving the compound in DMSO. After 24 hours and 48 hours of treatment, dishes were treated with trypan blue for cell death counting. Bars here represent mean \pm SD. a) The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=4$ independent experiments, one way Anova. b) The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=4$ independent experiments, one way Anova.

3.3.3 Glutathione levels in PC-12 cells after phthalate exposure

Since ROS production was reported earlier, it was interesting to investigate glutathione levels. This further information could give us an understanding about glutathione and if it is used under phthalate toxicity. Two concentrations, the low concentration of 100 μ M and higher concentration of 500 μ M was chosen on the basis of the dose-relationship curve (see 3.3.1).

a)



b)

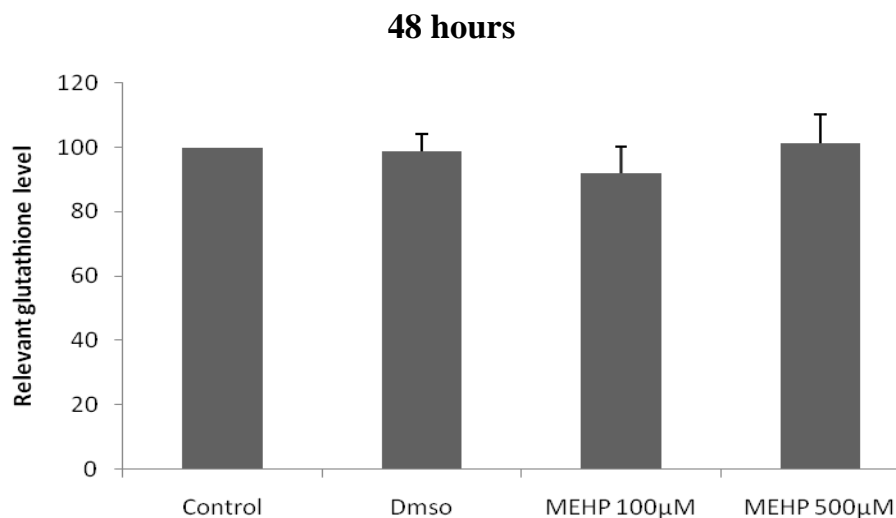


Figure 3.15: Glutathione levels at exposure of two different concentrations of phthalates in PC-12 cells. The dishes of PC-12 cells were treated on day two after the first day of splitting. After incubating them with additions of phthalates for 24 hours and 48 hours we have harvested the cells for the glutathione measurement. Glutathione measurement was completed and correlated for the protein. Bars represent mean \pm SD a) There is no significant treatments. Bars represent the median in this case. There is not a statistically significant difference ($P > 0.05$), where n is two independent experiments. b) The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P > 0.05$), where $n=4$ independent experiments, bars represent mean \pm SD, one way Anova.

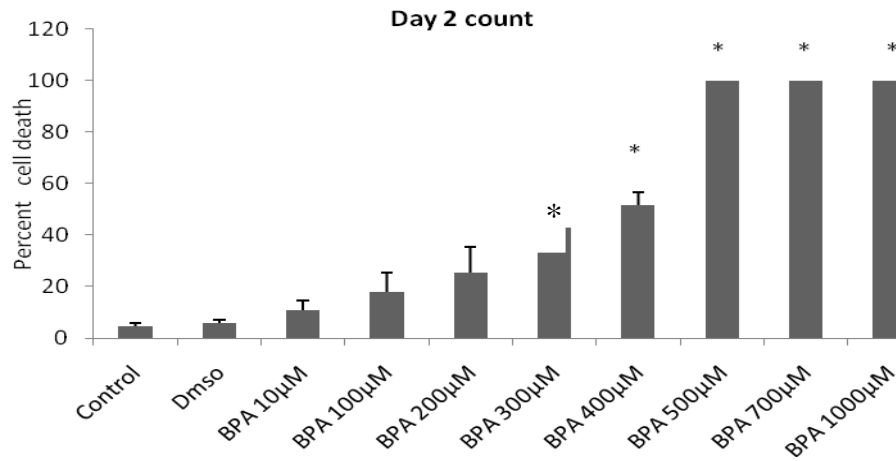
3.4. Chicken Neurons

Chicken neurons were used as a different model from PC-12 cells, to study the toxicity of bisphenol-A. Cell death count was calculated on the exposed dishes.

3.4.1 Dose-response in chicken neurons under bisphenol-A exposure

Chicken neurons were treated with bisphenol-A on different days of neurons maturation in vitro. First day chicken neurons were treated and counted on the second day. The other chicken neurons were treated with bisphenol-A on the third day and counted on the fourth day. The two time points were chosen to see if there would be any difference in the toxicity of bisphenol-A with the time treated. The results showed a dose-response (Fig 3.16) Treating dishes with higher concentration than 400 μ M bisphenol-A (BPA) gave 100 percent death in chicken neurons. The results also showed that the treatments 300 μ M bisphenol-A and above concentrations were significant when dishes were treated on day one and counted on day 2. The data from the day 4 (Fig. 3b) showed an increase in the cell death when comparing with day 2. The concentration 200 μ M and all the above concentrations were significant for the day 4.

a)



b)

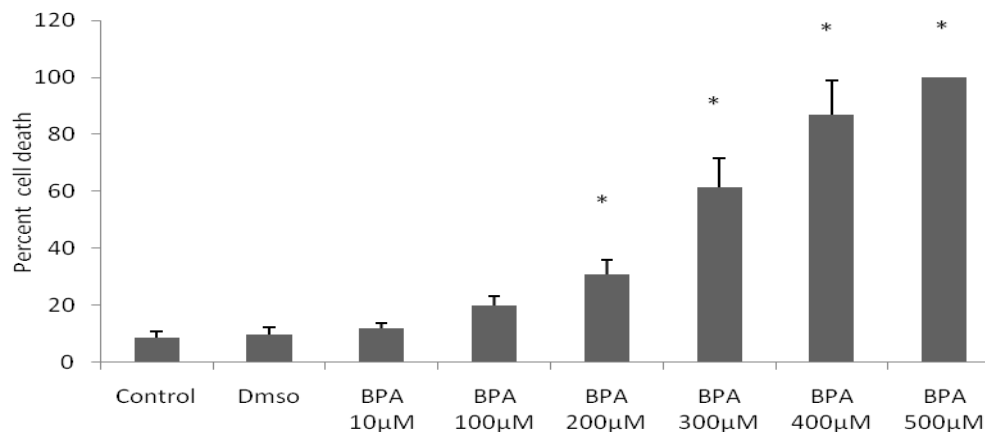
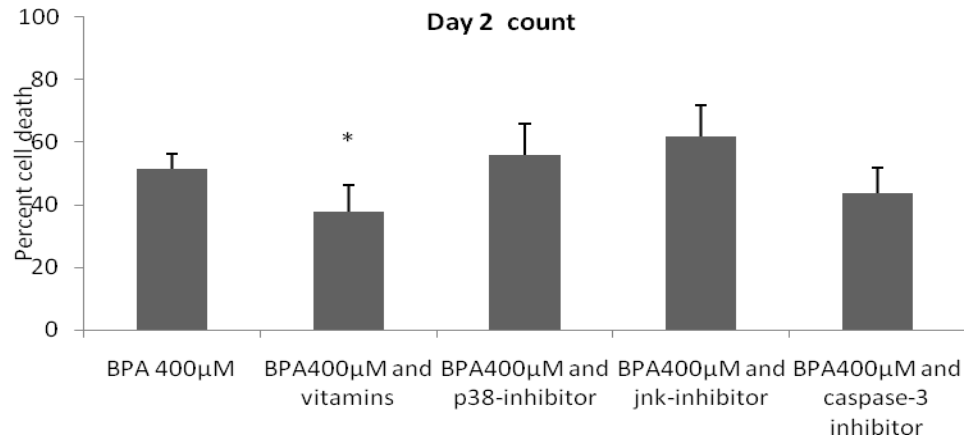


Figure 3.16: Dose-response in chicken neurons under bisphenol-A exposure. Chicken neurons have been exposed to different concentrations of bisphenol-A. Bisphenol-A have been dissolved in DMSO. The dishes were treated on day two with bisphenol-A concentrations. Chicken neurons dishes then were treated with trypan blue after 24 hours (a) and after 48 hours (b) of treatment. Cell death data was collected. a) The chicken neurons were treated on the day one and cell death count was performed on the day two with trypan blue staining. There are 6 dishes from 3 independent experiments. Cell death average was calculated and expressed as mean \pm SD. The results from 300µM concentration of bisphenol-A and above are significant ($P < 0.05$), one way Anova. b) The chicken neurons were treated on the day three and cell death count was performed on the day four with trypan blue staining. There are 6 dishes from 3 independent experiments. Cell death average was calculated and expressed as mean \pm SD. The results from 200µM and above are significant ($P < 0.05$), one way Anova.

3.4.2 Mechanisms of Bisphenol-A toxicity

Since it is reported that ROS is generated under the bisphenol-A toxicity (Bindhumol et al., 2003), it was interesting to find the mechanism by which bisphenol-A affect the cells signaling. MAP-kinase inhibitors p38-inhibitor, jnk-inhibitor and caspase-3 have been chosen for this experiment. Earlier literature suggests that caspase-3 is involved in the death pathway under exposure of bisphenol-A (Brown, 2009). Therefore these inhibitors are the main focus in this study. Vitamins as neuroprotective agents were also administered under the bisphenol-A toxicity to see if they could give any protection. The results from the day 2 and day 4 are similar. At the day 2 of counting, the cell death of the treatment with bisphenol-A 400 μ M together with vitamins is significant (Fig.3.17a). Treatment with vitamins gave less cell death count on the day 2 than day 4, when comparing with other treatments. T-test was performed, and with P less then 0.05 the treatment at day 4 was significant. Therefore it was possible to conclude that cell death was higher at the day 4.

a)



b)

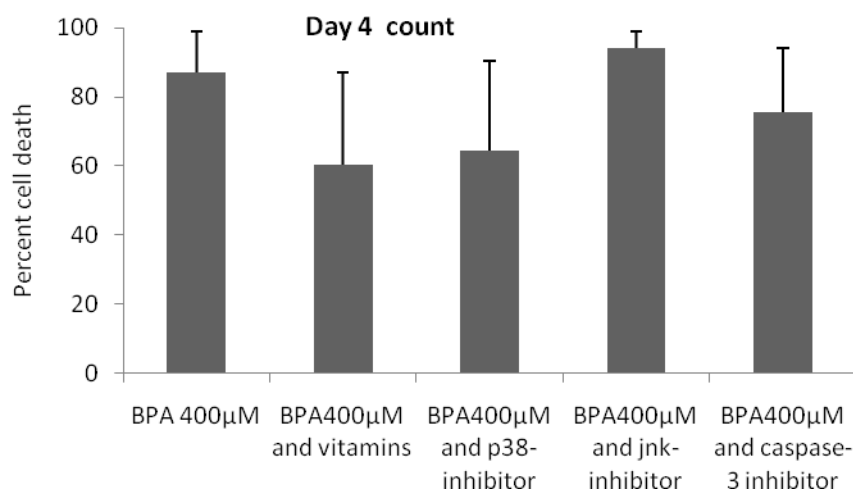


Figure 3.17: Mechanisms behind bisphenol-A toxicity. Chicken neurons have been exposed to 400µM bisphenol-A with different inhibitors. a) The chicken neurons were treated on the day one and cell death count was performed on the day two with trypan blue staining. There are 6 dishes from 3 independent experiments. Cell death average was calculated and expressed as mean \pm SD. Vitamins together with bisphenol-A at 400µM is significant and $P < 0.05$, where n is 6 dishes from three independent experiments, Dunnett's method; b) The chicken neurons were treated on the day three and cell death count was performed on the day four with trypan blue staining. There are 6 dishes from 3 independent experiments. Cell death average was calculated and expressed as mean \pm SD. There is no significance between the treatments ($P > 0.05$), one way Anova.

4. Discussion

4.1 Discussion of cell models of study

4.1.1 Use of PC-12 cell line to study neurons

PC-12 cells is a practical model to study the cell death machinery and neurobiology (Tischler et al., 1983). PC-12 cells in current research have been used without NGF (nerve growth factor). This was done for PC-12 cells in order to avoid neurite outgrowth. Withdrawal of NGF causes PC12 cells to have distinct and well-defined apoptosis (Xia et al., 1995). Such apoptosis is similar to apoptosis of sympathetic neurons (Xia et al., 1995) NGF is critical for PC-12 survival in serum-free media, and it initiates neuronal differentiation via interaction with NGF receptor further activating genes in PC-12 cells (Batistatou et al., 1992). MAP kinase involvement was also identified in PC-12 cells (Gomez et al., 1992). This model is a useful model to study the neurotoxicity of different compounds and to investigate how chemicals like bisphenol-A and phthalate affect MAP kinase. PC-12 cells without NGF are under constant activation of jnk, p38 and inhibition of erk at the time of the initiation of apoptosis (Xia et al., 1995). Erk is activated by growth factors while jnk and p-38 are activated by environmental stress (Xia et al., 1995). Activation of erk is involved in proliferation and differentiation, while activation of jnk and p38 is involved in cell death (Xia et al., 1995). Caspase-3 is also identified through the Western blotting procedure, where cytochrome c release together with caspase-9 activates caspase-3 when PC-12 cells are treated with H₂O₂ (Yamakawa et al., 2000). Caspase-3 activity is reported under deprivation of PC-12 cells (Kim et al., 1999). This implicates beginning of apoptosis through the release of ROS in oxidative stress. ROS production is reported in serum-deprivation of PC-12 cells (Sato et al., 1996). Therefore this model was useful for the designed study the effect of environmental disruptors.

4.1.2 Use of cerebellar chicken neurons to study real neurons

The first model for studying the neuronal differentiation and apoptosis was cerebellar granule cells from rat (Vaudry et al., 2003). Caspase-3 and MAP kinase activities are identified in the cerebellar granule rat neurons (Vaudry et al., 2003). Caspase-3 is involved in the cell death machinery breaking down components like actin, Bcl-2 and PARP (poly-ADP ribose polymerase) in the cerebellar granule rat neurons. Glutamate is believed to be a neurotransmitter for cerebellar granule neurons (Gallo et al., 1982). Granule neurons also depend upon K^+ concentration in the medium. High K^+ concentrations increase granule neurons survival, and Ca^{2+} voltage- channels are involved in granule neurons survival, on the contrary high activity of these channels lead to neuronal degeneration (Gallo et al., 1987).

Neurons also express NADPH oxidases: NOX1, NOX2 and NOX4. It was documented earlier that initiation of apoptosis in neurons due to serum-deprivation is initiated by NOX2 (Bedard and Krause, 2007). NOX enzymes play a central role in intracellular signaling of the central nervous system (Bedard and Krause, 2007). NOX2 is thought to play a major role in the activation of the neurodegeneration and microglia that leads to diseases like Parkinson's disease, Alzheimer's disease, demyelinating diseases and dementia (Bedard and Krause, 2007). Thus NOX family NADPH oxidases plays an important role and is responsible for many assignments in the cellular environment (Bedard and Krause, 2007). Cerebellar granule chicken neurons are chosen on the basis that ROS production and caspase-3 activation is reported during apoptosis (Jacobs et al., 2006). It was interesting to have chicken neurons as an alternative parallel model to PC-12 cells. Unlike PC-12 cells, chicken neurons represent real neurons, and the maturation of neuronal cells could be implicated in the study of environmental disruptors at different stages of neurons maturity. The results can possibly reveal that exposure of environmental chemicals like bisphenol-A and phthalate may depend on developing stages of neurons

(Fig.3.16). For example, day four showed higher death in chicken neurons under bisphenol-A exposure then day two.

4.2 Effect of antioxidants on glutathione levels in PC-12 cells

4.2.1 Effect of vitamins on glutathione level in PC-12 cells

The combination of vitamin E and C is found to inhibit apoptosis in human endothelial cells (Borek, 2004). Vitamin E is also found to activate transcription factor NF- κ B which is involved in cell survival in rat cerebellar granule neurons (Behl, 2000). These vitamins decrease the activity of pro-apoptotic Bax and increase activity of Bcl-2. Vitamin E gives a great protection to neurons by preventing production and accumulation of ROS (Borek, 2004). Vitamin E, vitamin C and glutathione promote survival of neurons in vitro and in vivo (Ravati et al., 2000). As well vitamin E and estrogens protect against glutamate, Fe^{2+} and amyloid β peptide through the reduction in lipid peroxidation (Ravati et al., 2000). In current study the combination of vitamin C and vitamin E have been used, but glutathione levels did not show any significant changes. There was a tendency for vitamins to protect against reduction in reduced glutathione at a later stage after 48 hours (see fig.3.3), but the results were not significant. Use of vitamins as ROS scavengers was later implicated in this study on bisphenol-A and phthalate (MEHP) toxicity that would be discussed later.

4.2.2 Effect of substitution of endogenous glutathione by additional exogenous glutathione on glutathione level in PC-12 cells

Much evidence suggests that exogenous glutathione increases GSH level and prevents cytotoxicity of serum-deprived PC-12 cells (Lee et al., 2002). In current study glutathione ester was used. Glutathione ester protects against radiation, carcinogens and heavy metals while increasing the activity of glutathione transferase, glutathione peroxidase and glutathione reductase (Świdarska-Kołacz et al., 2008). Since GSH is used by ROS species, as discussed earlier, it was interesting to see if treatment with glutathione ester could substitute the lost GSH. Addition of glutathione to PC-12 cells with serum does not affect γ -GCS promoter, but in the serum-deprived PC-12 cells, the trend of down-regulation of promoter is hypothesized, but still is not significant enough to make any further conclusions (Øverby et al., 2008). Exogenous glutathione ester treatment did not show any significant effect on the glutathione levels in this research. Extra addition of glutathione in PC-12 cells with serum has shown significant decrease in the 24 hour point. This is contradictory to the expected increase in GSH. In this study concentrations of 2.5mM and 5mM were used. The study on PC-12 cells shows that treating cells with 10mM of exogenous glutathione in serum deprivation, increases cell viability (Lee et al., 2002). On the contrary, addition of 3.25 μ M exogenous glutathione concentration could protect SK-N-SH human neuroblastoma cells from cell death only and β AP (β -amyloid) toxicity only in the presence of α -estradiol (Gridley et al., 1998). This is explained by synergistic interaction between α -estradiol and GSH, which does not depend on estrogen-receptor mechanism (Gridley et al., 1998). The concentrations chosen in this experiment might have not been high enough to effect glutathione level. The negative findings in current research of decreased glutathione can further be argued by fact that glutathione ester have been used and not real glutathione. Glutathione synthesis and pathways are very complicated and are controlled by negative feedback mechanism (Griffith, 1999). More experiments have to be conducted to infer the cause for the low

levels of GSH when treated with extra glutathione. And since glutathione is a negative feedback mechanism, the concentrations could also been high enough to trigger opposite pathways.

4.2.3 Effect of α -estradiol in the serum-deprived PC-12 cells

Current research on glutathione levels in PC-12 cells under exposure to estrogen like α -estradiol in serum-free medium showed reduced levels in GSH. This contributes and supports the evidence found in the study by Gunn Rebecca Øverby of 2008. The down-regulation of promoter γ -GCH is revealed under PC-12 cells that are deprived along with α -estradiol. The mechanisms behind this down-regulation are not yet well understood, but it is likely that GSH decreases as a result of α -estradiol effect of GSH synthesis, by that affecting the γ -GCS promoter. The decreased levels of glutathione are associated with cell death and increase in calcium levels within PC-12 cells (Jurma et al., 1997). Intracellular calcium is important for sustaining the membrane and its membrane potential (Wang et al., 2006). The reduction in GSH alters dopaminergic neurons that serve as neurotransmitter system to change the calcium homeostasis (Jurma et al., 1997). As a result of calcium imbalance ATP is depleted and the excess calcium blocks the membrane potential (Wang et al., 2006) These can be the mechanisms involved in deprived PC-12 cells with α -estradiol, but further research needed to promote a detailed overview of consequent molecular actions.

Apoptosis can be triggered through the MAP kinase: erk, jun and p-38 (Bedard and Krause, 2007). In this research it was found out that p38 is involved in decreasing glutathione when PC-12 cells are deprived with α -estradiol. It is known that programmed cell death of p-38 MAPK pathway gets activated when the ROS production has started (Bedard and Krause, 2007). This pathway is said to be started due to the activation of erk1/erk2 pathway or due to the inhibition of the phosphatase (Bedard and Krause, 2007). The tendency of p38 pathway involvement in the deprivation with α -estradiol has been noticed. Even though data was not significant p38 involvement in this situation is hypothesized. More experiments should be conducted to uncover the mechanisms behind.

4.3 Bisphenol-A toxicity in PC-12 cells and Cerebellar Granule Neurons

4.3.1 Effect of Bisphenol-A

MAP kinase starts due to the ROS-dependent inhibition of tyrosine phosphatase (Bedard and Krause, 2007). Central nervous system is very sensitive to ROS because of polyunsaturated fatty acids that are contained in membranes (Bedard and Krause, 2007). It is also reported earlier that low levels of GSH leads to mitochondrial function decreases, further translocating the apoptosis inducing factor (AIF) and then cytochrome c release activated caspase-3 (Chi et al., 2007). BPA is thought to accumulate in mitochondrial membrane and restrain complex I activity by process of oxidative phosphorylation (Ooe et al., 2005). Dose-response to bisphenol-A corresponds to that the higher concentration gives higher cell death in both PC-12 cells and the cerebellar granule chicken neurons. Toxicity after 24 hours exposure to bisphenol-A in both cases was clearer, than after 48 hour exposure. Middle concentrations increased cell death efficiently in PC-12 cells (Fig.3.10) and were more toxic to granule neurons (Fig. 3.16).

4.3.2 Uncovering the mechanisms behind bisphenol-A toxicity

Bisphenol-A mimics neurotoxic actions of estrogen in developing cerebellar neurons (Le et al., 2008). Bisphenol-A also alters dendritic and synaptic development in cultured hypothalamic neurons of fetal rats (Iwakura et al., 2010). Low doses of bisphenol-A forces Ca^{+} signaling via non-genomic pathway in hippocampal neurons (Tanabe et al., 2006). Extremely low concentrations of bisphenol-A interfere with normal gene expression (Quesada et al., 2002). Bisphenol-A activates new type of estrogen receptor on the cell membrane (Quesada et al., 2002). Alteration of gene

expression through activation of CREB (cAMP response element-binding) is reported under bisphenol-A exposure (Quesada et al., 2002). Genes like c-fos, tyrosine hydrolase and other neuropeptides regulate CREB signaling protein. CREB is involved in the survival of neurons (Ao et al., 2006). Constant CREB phosphorylation is vital for cell survival, while blocking CREB leads to neuronal degeneration in mammalian neuronal cells (Ao et al., 2006). Deletion of CREB in the genetically modified mouse leads to death of mouse embryo (Dawson and Ginty, 2002). In this research it was found that bisphenol-A reduces PC-12 cells survival in dose-dependent manner. The same dose-dependent neuronal death was observed in cerebellar granule chicken neuron cells. Dose-dependent death under bisphenol-A exposure is seen in the PC-12 cells and cortical neurons (Lee et al., 2007). Bisphenol-A is known to activate MAP kinase. Erk and jun are involved in the toxicity of bisphenol A in hippocampal neuronal cells (Lee et al., 2008). When looking at the graph of mechanisms that are involved in cell death under bisphenol-A exposure (3.11), the results show no significance. The concentration of 200 μ M of bisphenol-A was chosen for experiment along with inhibitors. If the concentration of BPA was higher than it would be possible to see more difference in the action of inhibitors as cell death increases. Looking at the graph (see fig.3.11), it is possible to say that there is a trend in vitamins C and E protection in PC-12 cells after 200 μ M BPA exposure, but this is not significant change. Vitamin E protects neuronal cells from the oxidative stress (Behl, 2000). Vitamin C also protects neurons, but Vitamin C co-administration with bisphenol-A on its own intensifies oxidative stress in rat's brain (Aydogan et al., 2008). Therefore it was interesting to see the effect of combination of these vitamins on BPA on PC-12 cells. There is a tendency for vitamins C and E to protect against bisphenol-A in PC 12 cells, but this tendency is not significant. Evidence show that immediate treatment with antioxidants protects from outburst of ROS (Ravati et al., 2000). The experimental results on cerebellar granule chicken neurons showed that vitamins C and E give protection to the neurons at the earlier stage of neuron development. This protection is due immediate treatment which

stopped the ROS outburst. The caspase-3 inhibitor was also used in this study. Caspase-3 activity is registered at the BPA exposure in rat's neurons (Brown, 2009). Caspase-3 is also seen to be activated in neuron and glia cultures from the mouse (Miyatake et al., 2006). In the current experiment, there is a tendency of caspase-3 activity after bisphenol-A exposure in PC-12 cells. Similar experiment but with higher concentration of BPA should be done in the future, and one can investigate if vitamins C and E have a neuroprotective action against BPA toxicity. Caspase-3 activation is hypothesized, therefore more experiments should be performed but at a higher concentration of BPA. When considering the data for cerebellar granule chicken neuron it was possible to see that vitamins C and E have protected against BPA toxicity at 24 hour point. In this current study of BPA toxicity on chicken neurons, higher concentration of BPA was used at 400 μ M BPA. At 48 hour point vitamins have not been efficient for further protection of the chicken neurons. The same inhibitors used in the PC-12 cells have been used for the study of chicken neuron cells. Other trends of protection by any other inhibitor were not detected. Cerebellar granule chicken neurons and PC-12 cells could possibly have different mode of pathways after BPA exposure, but much more studies have to be inferred in order to investigate possible mechanisms.

4.3.3 Effect of bisphenol-A on glutathione levels

ROS sensitive cystein residues are the one that counter attack the cell functions (Bedard and Krause, 2007). The signal transduction is also said to be effected by the ROS which reduce phosphatase activity by that managing the activity of protein tyrosine phosphorylation which majorly controls the signal transmission (Bedard and Krause, 2007).

Bisphenol-A induces ROS production (Lee et al., 2008). When ROS is induced in the serum-deprived PC-12 cells, glutathione levels falls down. Glutathione can be either

used or synthesized. When deprivation decreases glutathione levels, we assume that ROS uses endogenous glutathione. In case of bisphenol-A, there is no major changes in glutathione levels of PC-12 cells. Glutathione levels do not change; therefore we can assume that there is another ROS mechanism behind the bisphenol-A toxicity. At the same time there is evidence that bisphenol-A decreases glutathione levels. BPA induces overproduction of hydrogen peroxide in the mouse brain, kidney, liver and other organs (Kabuto et al., 2003). Glutathione levels of GSH are decreased in the brain of rats treated with bisphenol-A (Aydogan et al., 2010). Overall it can be inferred from results of current research that bisphenol-A toxicity involves another type of ROS then ROS which is produced by deprivation.

4.4 Effect of phthalate toxicity in the PC-12 cells

4.4.1 Effect of MEHP in PC-12 cells

MEHP is considered to have such dose-response where lower doses can cause more harmful effects than the larger doses of chemicals, this is called a non-monotonic response (Andrade et al., 2006). In the current study, the dose-response has increased with higher concentration respectively in PC-12 cells. As mentioned earlier the cell death does increase with MEHP concentration in PC-12 cells, but estrogenic qualities of such an environmental disrupter could be more harmful if it has other gradual effects on the cell signaling and development. Even though both Bisphenol-A and phthalates are estrogenic, and they are different in effects on Ca^{2+} . Bisphenol-A has Ca^{2+} driven from the cells, while MEHP is responsible for Ca^{2+} influx into the cells (Palleschi et al., 2009). These calcium disruptions further lead to disturbances in cell signaling.

4.4.2 Uncovering the mechanisms behind phthalate toxicity in PC-12 cells

MEHP (mono-2-ethylhexyl) phthalate induces apoptosis and activation of caspase-3 in U937 cells (Yokoyama et al., 2003); (Lim et al., 2009). Evidence suggests that phthalates firstly target and damage mitochondria (Onorato et al., 2008). MEHP toxicity implicates damage to mitochondrial function by the release of cytochrome c (Kasahara et al., 2002). MAP kinase is involved in MEHP toxicity, especially activation of phosphorylation of p38 (Rakkestad et al., 2010). Since there is evidence of MAP kinase and caspase-3 involvement in apoptosis, the inhibitors for these pathways were implicated in the experiment. Vitamins C and E as ROS scavenging antioxidants were of interest. Earlier it was found that the combination of vitamins E and C gives a tendency to protect against cell death in the PC-12 cells (see 4.3.2). The operating concentration for MEHP in PC-12 cells was chosen at 500 μ M. The cell death count at this concentration was low, and therefore affected the difficulty in interpreting the results. The results on the graph (see 3.14) do not show any significance. Trend of vitamins protection and jnk involvement in apoptosis is hypothesized from the results at 24 hour point. At 48 hour point vitamins do not show any significant decrease, but tendency of jnk and caspase-3 involvement in toxicity is theorized. There are few studies that describe phthalate toxicity on neurons and PC-12 cells. The estrogenic characteristic of such compound makes it difficult to evaluate and find correspondence between the toxicity and the pathways.

4.4.3 Effect of phthalates on glutathione levels in PC-12 cells

Since ROS production believed to use glutathione, as in deprivation, it was interesting to investigate glutathione levels under MEHP exposure. Glutathione levels have not decreased as expected, and therefore we inferred that there is another ROS mechanism behind phthalate toxicity. One again investigating environmental estrogen

disruptors proves difficulty in uncovering the mechanisms behind its toxicity. Non-genomic way of estrogens and cell signaling are not well understood in molecular biology (Jeng et al., 2009).

4.5 Constrains and evaluation of Bisphenol-A and Phthalate toxicity

Estrogens like bisphenol-A, phthalate (MEHP) and α -estradiol are compounds that are difficult to study as they involve non-genomic pathways and non-monotonic dose-response (Watson et al., 2010). Low doses of estrogens might be more harmful in the gradual accumulation of it in the cells than with higher doses (Quesada et al., 2002). Therefore it is extremely hard to extrapolate any regularity of such environmental disruptors. Deprivation of PC-12 cells induced apoptosis through production of ROS and reduction of glutathione levels. These findings are different from toxicity of estrogen disruptors. Since ROS production is reported in the toxicity of these chemicals, the expected glutathione levels did not decrease under bisphenol-A and MEHP exposure. This suggests a possible different ROS production from ROS in the deprived PC-12 cells. Different mechanisms and multiple negative mechanisms and pathways could control the expression of certain genes in PC-12 cells as well as in cerebellar granule chicken neurons. Since it was discussed earlier that concentrations of chemicals were too low for inducing cell mortality, the concentrations should be increased for the further studies. Gradual exposure to these chemicals should also be a priority as lower concentrations do effect the alteration of gene expression which higher concentrations do not (Tanabe et al., 2006). “Whole mixture approach” entails studying the combinations or mixtures of environmental estrogens (Kortenkamp, 2007). It suggests the approach of additivity which entails evaluation of estrogenic compounds in combination. Since human-beings and organisms are exposed to a

mixture of estrogenic compounds at low doses, it might be useful to study the “estrogenic cocktail” (Kortenkamp, 2007). Actions of estrogens alone can be higher or lower than the sum of actions of estrogens together (Kortenkamp, 2007). It is important to take into consideration, that compounds that have estrogenic potency have a cumulative action, this is why it might be of more importance measuring the total estrogenic accumulation due to environmental contamination (Jobling et al., 1995).

4.6 Conclusion

1. Effect of deprivation, bisphenol-A and MEHP involves different ROS and different mechanisms. Glutathione levels after exposure to estrogen disruptors did not show a decrease such as seen in the deprived PC-12 cells
2. Glutathione ester did not substitute for endogenous glutathione during serum deprivation, therefore putting emphasis on possible substitution of glutathione ester by another form of glutathione
3. Vitamins C and E showed protection at early maturation of neurons in cerebellar granule chicken neurons after bisphenol-A exposure. There is a tendency of vitamins protection and caspase-3 involvement under bisphenol-A exposure in PC-12 cells.
4. Vitamins C and E, jnk and caspase-3 inhibitors showed tendency to protect against phthalate toxicity at different time points, but concentrations have to be increased in further studies.
5. Connection between the decrease in glutathione levels and decrease in γ -GCS promoter is established in deprived PC-12 cells treated with α -estradiol

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Chemicals and biological products

Product	Producer
BCA	Sigma-Aldrich Co.
BPA	National Health Institute, Oslo
BME	Invitrogen Co.
BSA	Sigma-Aldrich Co.
CLB	Sigma-Aldrich Co.
DMEM	Invitrogen Co.
DMSO	Sigma-Aldrich Co.

DTNB	Sigma-Aldrich Co.
α -estradiol	Sigma-Aldrich Co.
Etanol, absolute prima	Arcus products
FBS	Invitrogen Co.
Glutathione	Sigma-Aldrich Co.
HS	Invitrogen Co.
MEHP	National Health Institute, Oslo
SSA	Sigma-Aldrich Co.
A-Tochopherol	Sigma-Aldrich Co.

Trypan blue	Sigma-Aldrich Co.
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